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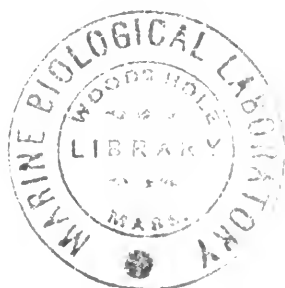
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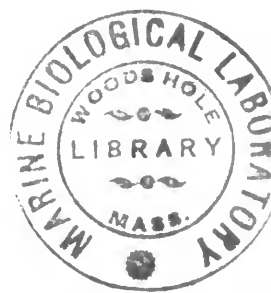
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IMMUNOLOGY



IMMUNOLOGY

BY

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SECOND EDITION

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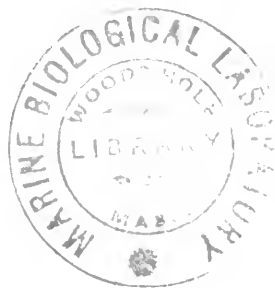
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TO MY WIFE
THIS BOOK IS MOST
AFFECTIONATELY DEDICATED

PREFACE TO SECOND EDITION

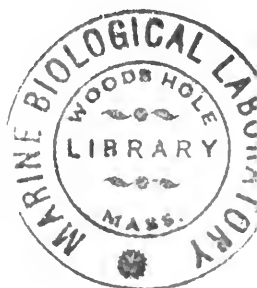
In preparing this edition of the book the author has attempted to bring the subject matter as nearly up to date as possible and to rearrange the material so that it can be outlined by the student more readily. This has necessitated rearrangement of the chapters as well as of their contents in many instances. Where it has been deemed advisable, material has been deleted. Two new chapters, one on the reticulo-endothelial system and the other on serum reactions, have been added. The chapter on colloids has been placed in the Appendix where it can be referred to by students unfamiliar with the elementary chemistry of colloids. The chapters dealing with the serology of syphilis have been revised in accordance with the procedures approved by the "Committee on the Need of Adherence to Conventional Technique in the Performance of Reliable Serologic Tests for Syphilis" appointed by the Surgeon-General of the United States Public Health Service.

The author is very appreciative of the helpful suggestions given him by Dr. K. Landsteiner, Dr. W. J. Nungester and others working in the field of immunology. The many constructive criticisms given by reviewers of the first edition have been of considerable help in this revision.

As in the preparation of the first edition, a number of colleagues and members of my family have rendered material assistance in the preparation of the manuscript of this revision. Among these, Dr. C. M. Downs, Mr. Harold Nelson, and my wife have been extremely helpful.

NOBLE PIERCE SHERWOOD.

Lawrence, Kansas.



PREFACE TO FIRST EDITION

This book is written for medical students and for others who have had training in pathogenic bacteriology, inorganic and organic chemistry and who are interested in the underlying principles involved in infection, resistance, and diagnostic laboratory tests. It has been used in mimeograph form at the universities of Kansas and Montana, respectively, for the past few years. From this experience we have gained the impression that medical students and college students majoring in bacteriology are able to read and discuss intelligently the subject matter as it is here presented. It is assumed that the text will be supplemented by laboratory experiments since it is difficult and perhaps impossible to gain a working knowledge of the subject without observing many of the phenomena discussed.

The author has attempted to lead the student to correlate some of the teachings of physiology, pharmacology, organic, biological and physical chemistry as well as anatomy, pathology and general biology and apply these teachings to the elucidation of the mysteries surrounding infection, resistance, and diagnostic procedures. He has attempted to show that clinical, experimental and preventive medicine contribute a great deal to our knowledge of the subject. In fact it is hoped that the student will acquire enough of the underlying philosophy of immunology to enable him to attack, with reasonable success, the practical problems he will encounter when he comes into contact with patients and the community at large in his clinical years in medicine. While empirical knowledge may enable one to perform laboratory or other immunological techniques, clinical experience and an understanding of the underlying mechanisms and extraneous factors involved is quite necessary to a correct interpretation of the results obtained.

In view of the fact that the student needs to acquire an adequate vocabulary of technical terms used in the text, definitions are given quite freely. A chapter on colloids is introduced just before the chapters that deal with the mechanisms involved in agglutination, opsonification, and complement fixation. This is done for the benefit of those students who are unfamiliar with colloid chemistry.

Throughout the book standard techniques are presented, analyzed, and discussed. It is hoped that reference to the practical application of immunological procedures will promote a greater interest in the subject on the part of the student.

The chapters on hypersensitiveness are placed last because an understanding of the material contained in the chapters preceding them is of material value in understanding the present theories pertaining to the complex problems inherent in allergy. A great deal of emphasis is placed upon the subject of specificity since it plays such an important part in diagnostic tests and in passive and active immunization. Since the material in many of the chapters is rather condensed, the lists of references at the ends of most chapters have been somewhat extended to accommodate the student who desires to read more extensively about some particular subject. For the convenience of the reader a synopsis of the three chapters on specificity is presented in a separate chapter (XV).

A number of colleagues, former students and friends have rendered material assistance in the preparation of the manuscript for this book. To all of them the author wishes to express his appreciation. There is space for mentioning only a few. Among these, Mr. Harold Clark deserves credit for outlining the chapter on flocculation tests in syphilis, in addition to rendering help in many other ways. Doctors C. M. Downs, H. R. Wahl, and Joel Wahlin have criticized parts of the manuscript. Dr. Ray Brewster and Mr. Morgan Rarick have rendered a great deal of assistance in the preparation of the chapter on modified antigens. Four other individuals whose services have made possible the completion of this volume are Miss Rosella Blood, the artist, Miss Letha Lemon and Mr. Harold Nelson, secretaries, and Mr. Louis Forman, who checked the bibliography.

The author has drawn quite freely from information contained in various publications. These are listed in the text, and an attempt is made to give due credit to both author and publisher.

NOBLE PIERCE SHERWOOD.

Lawrence, Kansas.



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IMMUNOLOGY

IMMUNOLOGY

CHAPTER I

INFECTION AND INFECTIOUS AGENTS

Infection.—The phenomenon of infection is usually considered one in which an infectious agent gains entrance to the tissues of a susceptible organism (the host) and finds suitable conditions for growth and development in its new environment.

CLASSIFICATION OF PARASITES.—These agents show wide variation in their habitat, size, shape, chemical composition, and physiological functions. They are usually classified into groups such as (1) animal parasites, (2) pathogenic fungi, (3) pathogenic bacteria, and (4) viruses. These groups are not sharply marked off from each other, but each embraces many organisms of doubtful taxonomic standing.

Kendall (1923) divides the disease-producing bacteria into two main groups and discusses them under two headings: (1) the life cycle of parasitic bacteria; (2) the life cycle of pathogenic bacteria. The *parasitic* bacteria in contrast with the *pathogenic* are deficient in invasive power and in the ability to escape from the tissues. Both types of organisms are able to multiply within the tissues of the host after they gain entrance.

Goodpasture (1936) suggests that infectious agents be classified as extracellular, facultative intracellular, and obligate intracellular parasites. The immunologist was in reality first interested in one of the virus diseases, smallpox, and many years later in bacterial infections. More recently he has extended his interest to infections caused by members of the first two groups.

SYMBIOSIS.—After infection has occurred, it may or may not disturb the tissues of the host to any great extent, and in some instances both parasite and host may be benefited. In the case of root-nodule or nitrogen-fixing bacteria that produce tumorlike growths on the roots of leguminous plants, the host is dependent upon them for simple nitrogen compounds necessary for the life

of the plant, while the bacteria are supplied certain food materials by the host. This relationship of mutual helpfulness is spoken of as *symbiosis*.

INFECTION WITHOUT PATHOLOGICAL CHANGE.—A generalized infection of the wild rat with *Trypanosome lewesi* or with *Leptospira icterohemorrhagiae* is not beneficial to the host but apparently does not elicit any physiological or anatomical disturbances that can be designated as infectious disease. In both of these examples of infection the organisms enjoy a wide distribution in the body and are present in great numbers in the blood.

INFECTION WITH PATHOLOGICAL CHANGE.—These examples of infection without pathological change or infectious disease are in marked contrast to what occurs when the *leptospira*, mentioned above, gains entrance to the tissues of another rodent, the guinea pig, or for that matter into the tissues of man. In either case the presence of generalized infection causes profound physiological and structural disturbances, frequently resulting in death.

TYPES OF PATHOLOGICAL CHANGES.—It would seem that if an infectious agent is to produce disease, it must not only gain entrance to the tissues and be able to live and multiply there, but it or its products of growth must be incompatible with the normal physiological functioning of the host and lead to discernible pathological (abnormal) changes. These may be local, focal, or general. For example, organisms gaining entrance to the body are frequently "filtered out" by the regional lymph glands. This may occur without any symptoms other than a slight increase in size and change in consistency of the glands. The organisms may be destroyed or remain quiescent for long periods of time or until conditions become favorable for their growth and dissemination.

LOCALIZED AND GENERALIZED INFECTION.—A wound may become infected and show abnormal changes such as redness, swelling, local heat and pain, without general physiological symptoms. As long as the infectious agents remain at the site of injury, it is a *localized* infection, but when they multiply and invade the blood stream, it becomes a *generalized* infection. This usually calls forth definite symptoms even before the blood stream is invaded.

BACTERIEMIA, SEPTICEMIA, AND PYEMIA.—If bacteria gain entrance to the blood stream from some focus but are unable to multiply in the circulation, the condition is spoken of as a

bacteremia. If multiplication occurs in the blood stream, it is called *septicemia* unless multiple abscesses develop, when the term *pyemia* is used.

ACUTE AND CHRONIC INFECTIONS.—Infections are often classified according to the duration of the disease. Those that disappear within a few days or at most a few weeks are called *acute* infections, while those that persist are either *subacute*, or *chronic* infections.

FOCAL INFECTIONS.—Chronic infections causing pus pockets around the roots of the teeth and inflammatory processes in the tonsils, sinuses, prostate gland, cervix of the uterus, or elsewhere are called *focal* infections. The detection and eradication of such foci of infection is regarded by most physicians as good medical practice. A critical appraisal of their importance in systemic disease is given by Reimann and Havens (1940). They seem to feel that an overemphasis has been placed upon the importance of focal infections in systemic disease and that surgical interference is frequently detrimental to the patient.

BENEFICENT INFECTIONS.—There are a few infections in man which, when viewed in one way, may be regarded as beneficent although not without danger in themselves. For example, consider a patient suffering from syphilis, which is caused by *Treponema pallidum*. He may have extensive infection of the tissues, including the brain, and develop spastic paralysis and insanity. This condition resists the ordinary treatment of syphilis, but if the patient should become infected with the malarial parasite, a clinical cure is possible. After his symptoms of syphilis have disappeared, he still has malaria, which can be treated and usually cured with quinine.

SPECIFIC MICROBIC ASSOCIATION.—Until recently it has been thought that each infectious disease was caused by a single specific infectious agent. Now it appears that swine influenza *may* be an exception to this concept. Shope (1931) and Lewis and Shope (1931) seem to have shown that this disease is due to simultaneous infection with two agents, one filtrable and a second that belongs to the hemophilic group of bacteria. This would seem to be an example of an infectious disease caused by specific microbic association where both microorganisms may be regarded as primary agents. Zinsser and Bayne-Jones (1939) seem to regard these as examples of primary (virus) and secondary (hemophilic bacteria) infections.

METASTASIS.—During the period of existence of a focal infection or the course of an infectious disease or, for that matter, following major or minor operations, there may occur new foci of infection in parts of the body not primarily involved. This extension of organisms to new regions, which is called *metastasis*, may occur either by way of the blood stream or lymphatics. This explains the development of pneumococcus meningitis, typhoid meningitis, or tuberculous meningitis associated with the respective diseases. It likewise explains bone involvement (osteomyelitis) and inflammation of venous walls (phlebitis) during typhoid fever. Patients operated upon for localized streptococcus infections in the abdomen not infrequently develop phlebitis of the femoral or popliteal veins of the left leg. It is upon this basis that one commonly explains many cases of inflammation of the gall bladder (cholecystitis), kidney (nephritis), pancreas (pancreatitis), etc.

SECONDARY INFECTIONS.—Not infrequently a patient suffering from a specific infectious disease may develop a secondary infection due to an entirely different organism. The first disease lowered the patient's resistance and created a favorable soil for secondary invaders. One sees this occasionally in measles, which is apparently caused by a virus. These patients may develop a secondary streptococcus invasion of the lungs, which is called a streptococcus pneumonia. Secondary pneumonias of this type may be due to any of a number of organisms found upon the respiratory mucous membrane of the patient. In like manner secondary involvement of other tissues may occur and complicate the primary disease.

INFECTIOUS AND CONTAGIOUS DISEASES.—Any disease which is caused by an infectious agent is called an *infectious disease*. When it is quite readily communicated from one individual to another, it is classified as a contagious disease. Thus it is apparent that while all contagious diseases are due to infectious agents, not all infectious diseases are contagious.

Toxemic Diseases.—In a few diseases such as botulism, tetanus, gaseous gangrene, and diphtheria, infection either does not occur or is very slight. They are in reality toxemic in nature whereas in scarlet fever and in staphylococcus and pneumococcus infections there are both toxemia and severe infections.

BACTERIAL TOXINS.—Roux and Yersin (1888) were the first to show that some bacteria secrete toxins having specific actions in the

body and for which Behring and Kitasato (1890) have shown that antitoxins can be developed. Pathogenic streptococci also produce a substance, leucocidin, toxic for leucocytes. McLeod (1914) and Gay (1931) regard this as an important factor in the pathogenicity of streptococci. It is now generally agreed that each type of bacteria owes its specificity to its chemical constitution and in some cases to specific chemical products secreted by it. All diphtheria bacilli are not only more or less alike chemically and also chemically different from other bacteria, but they likewise all secrete a specific poison called diphtheria toxin. The true toxins differ from other poisons in that antitoxins are formed by the body in response to their presence within the tissues. One can then define a true toxin as an antigenic poison.

It has been customary to classify bacterial poisons into "*exotoxins*" and "*endotoxins*." The former are highly antigenic and are readily liberated from the bacterial cell while the "*endotoxins*" either fail to stimulate specific neutralizing substance or at best produce only a feeble response and are liberated with more or less difficulty from the bacterial cell. Eaton (1939) states, in effect, that a sharp line cannot be drawn between "*exotoxins*" and "*endotoxins*" because antigenic poisons exhibit all degrees of antigenicity and toxicity and also marked differences in the ease with which they can be separated from the bacterial cells. For a more extensive discussion of the subject the student is referred to Eaton's (1939) excellent review of chemical investigations of bacterial toxins and to the discussion of toxins by Zinsser, Enders, and Fothergill (1939).

BOTULISM.—In botulism the disease is due to a toxin formed by the bacteria outside the body, taken in with the food and absorbed through the mucous membrane of the intestine.

TETANUS.—In tetanus the organisms gain entrance to a wound or are mechanically carried into the tissues by a foreign body. If anaerobic and other satisfactory conditions prevail, the bacteria grow saprophytically and secrete tetanus toxin. This, according to Abels (1934), is carried to the central nervous system by the blood stream. The evidence, however, seems to indicate that the toxin is absorbed, probably through the end plates of the motor nerves, and reaches the central nervous system along their axis cylinders. When the toxin reaches the central nervous system, there develop certain physiological disturbances called tetanus.

GASEOUS GANGRENE.—Gaseous gangrene is not an infrequent complication of severely lacerated wounds. It is caused by *Cl. welchii* and a few other anaerobes and is characterized by necrosis (death) of the tissues infected and the liberation of gas within them. It has been quite definitely established that hemolytic streptococci, growing in gas-bacillus infected wounds, exalt the virulence of the anaerobes. In this we have another type of bacterial association.

DIPHTHERIA.—In diphtheria the organisms do produce a slight infection of the mucous membrane and grow luxuriantly in the inflammatory exudate. The disease is caused by the specific toxin secreted by the bacteria and absorbed into the general circulation. The disturbed physiological state is called diphtheria. Diphtheria toxin causes degenerative changes in the motor nerves, heart muscle, and vascular system.

ENDOTOXINS.—By means of extraction methods weakly antigenic endotoxins from members of the Salmonella, Proteus, and Colon groups have been obtained by Raistrick and Topley (1934), Boivin and Mesrobian (1937) and others. These endotoxins seem to be carbohydrate-lipid complexes although a second but weaker endotoxin, apparently a polypeptid, has been described. It has been known that Shiga dysentery bacilli produce a specific exotoxin. Boivin and Mesrobian (1937) made a chemical investigation of the toxins of Shiga dysentery bacilli. They report that the exotoxin, which is produced by both R. and S cultures, is a protein and acts on the central nervous system but not on the gastrointestinal tract. They were able to isolate endotoxins of a carbohydrate-lipid nature from both Shiga and Flexner dysentery organisms. These endotoxins produced symptoms of acute gastroenteritis in animals.

PHYTOTOXINS AND ZOOTOXINS.—The mycologist and the parasitologist have been more interested in morphological studies, life histories, and host-parasite reactions and relationships, than in chemical and physiological studies of parasites. A great many important physiological observations, however, have been recorded. Soule (1925) and Salle (1931) are pioneers in the study of the metabolism of protozoa. It is known also that the poisonous mushroom *Amanita phalloides* produces a soluble toxin whose properties have been studied by Ford (1910-11). Soluble toxins

have also been found in the seeds of *Ricinus communis*, *Croton tiglium*, and the bark and leaves of the locust tree (*Robinia pseudoacacia*). In the animal kingdom one finds toxins in the glandular secretions of a number of snakes, the ones most studied being those of the cobra, water moccasin, copperhead, and rattlesnake. Flexner and Noguchi found the venoms of the first two rich in neurotoxins but attributed the injurious effect of rattlesnake venom to hemorrhagin (vascular toxin).

It is not uncommon for a plant or animal to produce several toxins that differ in their affinity for different tissues. Perhaps the most common is a hemotoxin which destroys red blood cells. This type of toxin is found in arachnolysin (from certain spiders), ichthyotoxin (in eel serum), phrynosine (blood and skin of toads), in the poisonous secretions of certain fish (*Trachinus draco*), and in scorpions. These toxins of plant and animal origin are called "phytotoxins" and "zootoxins," respectively. For a more comprehensive discussion of the latter, the student is referred to a paper by Do Amarol (1928).

Antitoxins.—The antitoxins are specific neutralizing substances produced by the reticulo-endothelial system of the body and given off into the general circulation. Normal rabbit blood will not neutralize diphtheria toxin, but after the animal is given several small injections of toxin, it is found that the blood will neutralize the toxin, hence it is said that this blood contains diphtheria antitoxin. *Cl. tetani*, *Cl. botulinum*, *Cl. welchii*, and a few other anaerobes produce soluble toxins that show remarkable specificity. Recently Dick and Dick (1924) have shown that scarlet fever streptococci produce a soluble toxin responsible for the rash in scarlet fever. It is possible to purchase specific antitoxins for each of these bacterial toxins.

Ptomaines.—Other toxic substances are formed in culture media as the result of bacterial enzymes acting upon the food material. When enzymes act upon amino acids in such a way as to remove the carboxyl group (COOH) the process is called *decarboxylation* and the residue is an *amine*.

Toxic amines are called "ptomaines." Kendall (1931) has made the most extensive investigation of bacterial metabolism and has noted histamine production by a variety of organisms. Ptomaines are not only nonspecific but are produced by many saprophytes as well as parasites.

Pathogenicity.—The term *pathogenicity* is usually applied to the disease-producing property of an infectious agent.

VIRULENCE.—The term *virulence* has been used by many as synonymous with either the invasive power of an organism or its ability to multiply in the tissues or blood stream of a host. This definition seems inadequate for several reasons. In the first place, the term is applied to such toxicogenic organisms as *Cl. tetani* and *C. diphtheriae* as an index of their ability to form tetanus toxin and diphtheria toxin, respectively.

The “virulence test” of *C. diphtheriae* is essentially one for toxin production. *Cl. tetani* has no invasive power while *C. diphtheriae* invades the mucous membrane to a slight degree only. Both produce disease in suitable hosts by means of specific soluble toxins. In the second place, examples have just been given of infection without and also with pathological change. The host must be considered as a factor.

DEFINITION OF VIRULENCE.—It would seem appropriate to define virulence as the relative pathogenicity of an organism for a particular host. In examples previously cited it will be recalled that *Leptospira icterohemorrhagiae* is practically avirulent for the wild rat but highly virulent for guinea pigs and man. The same hosts exhibit similar resistance and susceptibility to diphtheria toxin.

MEASUREMENT OF VIRULENCE.—Virulence is usually expressed in terms of the amount of infectious agent that will, when properly administered, cause certain specified pathological changes in the host within a given time. There has been no uniform standard adopted for the measurement or dosage. Barber (1909), in describing the virulence of the anthrax bacillus for mice, recorded the actual number of organisms inoculated. Others have expressed the dosage of bacteria in terms of milligrams of growth obtained under standard conditions. The virulence of many bacteria has been described in terms of the least volume of a 24-hour broth culture grown at 37° C., or the fraction of growth obtained from a blood agar or plain agar slant grown under similar conditions, that will produce death or specified changes in the test animal. Some have even measured the dosage in terms of the standard platinum wire loop.

FACTORS ALTERING VIRULENCE.—Virulence may be diminished by frequent transfers in certain artificial media, culturing at temperatures above the optimum, or in some instances by desiccation. It may be increased, within limits, by growing the organisms on media containing blood or body fluids that favor capsule formation and also by animal passage. Pasteur showed that the passage of rabies virus through rabbits increased its virulence for the latter but diminished it for certain other animals. He found that exaltation of virulence, by animal passage, has a limit beyond which no increase could be obtained.

RELATION OF CAPSULE FORMATION TO VIRULENCE.—It seems that all pathogenic bacteria produce capsules when growing within the tissues of a host. It has been observed that when cultured outside the body the pneumococcus loses its virulence for white mice simultaneously with its ability to form capsules. Hence capsule formation is regarded as a factor in virulence.

HYPOTHESIS OF WELCH.—Welch offered an interesting hypothesis which bears his name. In this he postulates that the bacterial cell possesses a defensive mechanism which enables it to oppose the defenses of the body.

CORRELATION OF VIRULENCE AND COLONY TYPE.—Arkwright (1921) called attention to two important colony variations in a member of the colon-typhoid-dysentery group. These were the R or rough, granular colonies, and S or smooth, moist types. Both Arkwright and Baerthlein noted that S colonies might give rise to R colonies but that it was apparently more difficult for the process of dissociation to extend from R to S types. There is a tendency for colony types to remain stable but dissociation may occur spontaneously or may be induced. Intermediate between the R and the S is the O colony which has potentialities of both.

In the same year that Arkwright called attention to the existence of R, S, and O colony types, de Kruif (1921), working with colony variants of *Bact. leprosepticum*, found that cultures obtained from S colonies were virulent for rabbits while those obtained from R colonies were not.

The phenomenon of colony variation just described is called bacterial dissociation. It is now known that practically all bacteria may undergo dissociation and that there is a definite correlation between colony type and other biologic characteristics.

Among the pathogenic bacteria the S type is usually capsulated and virulent, while the R type is avirulent. An apparent exception to this occurs with *B. anthracis*. It should be noted, however, that in this case the R or rough colony is composed of capsulated organisms as are the virulent S forms of other bacteria and that the avirulent anthrax, although forming smooth S colonies, is said to be nonencapsulated. This is another example of the correlation of capsule formation with virulence.

LABILE ANTIGENS AND VIRULENCE.—In 1934 Felix and Pitt reported that virulent strains of *E. typhosa* possess a surface antigen not possessed by avirulent smooth strains. Because of its role in virulence they named it the "Vi" antigen of *E. typhosa*. It is destroyed by heat and most chemicals although formaldehyde is only slightly injurious to it. Perhaps one of the ways in which it confers virulence upon the bacteria is that it renders them more or less resistant to antibody action. Numerous reports have appeared in the literature stating that the strains of *E. typhosa* containing "Vi" antigen are more or less inagglutinable. Craigie (1936) discovered a bacteriophage specific for inagglutinable strains of *E. typhosa*.

The question of inagglutinability of strains of *E. typhosa* possessing "Vi" antigen has been investigated in this laboratory by Faucett (1940). He confirmed the results of others that the difference in agglutinability of resistant strains depends partly upon the "Vi" antigen content of the individual organisms. The strains of intermediate resistance are mixtures of resistant and sensitive strains.

Some doubt as to the correlation of Vi antigens and virulence has been raised by the studies of Robertson and Yu (1936) and of Kauffmann (1936). The former report that its presence is not primarily correlated with virulence of *Bacillus typhosus* while Kauffmann found that it was present and not correlated with virulence in *Salmonella paratyphi C*.

Mudd, Pettit, Lackmann, and Morgan (1939) have discovered a partially labile antigen in virulent streptococci. Its relationship to other specific substances and to virulence is not definitely determined. These findings suggest that perhaps labile antigens play an important role in bacterial virulence in general.

IMPORTANCE OF HOST FACTORS IN DETERMINING VIRULENCE.—Falk* (1928) has objected to the use of the term virulence “to denote the absolute capacity of a parasite to cause disease.” He points out that virulence is not primarily a characteristic of the parasite but “varies reciprocally as resistance or immunity.” In other words the relative resistance of the host is a factor. The *Leptospira icterohemorrhagiae* is not virulent for the wild rat, which is resistant to it, but is highly virulent for guinea pigs partly because they are very susceptible.

AGGRESSINS.—Bail attributed virulence to certain biochemical products formed either within the infected tissue or in cultures. He named these chemical substances “aggressins.” It has been shown that if *Cl. chauvei*, the causal agent of blackleg in cattle, is washed with sterile saline until freed of all traces of culture media and products of growth, it loses its virulence. The latter is restored, however, when the washed organisms are mixed with sterile filtrates of the original virulent culture. Scott (1931) has recently reviewed the literature on aggressins quite extensively.

Nature and Characteristics of Viruses.—Prior to 1935, very little was known about viruses other than that they are much smaller than bacteria, many being filtrable agents, and that they require living host cells for their reproduction; they cause specific diseases and recovery from such diseases is usually accompanied by a lasting immunity. From the standpoint of pathology it was well established that in many virus infections characteristic cell inclusion bodies are formed.

In 1935 Stanley obtained the virus of tobacco mosaic disease in crystalline form. While Stanley at first thought that the virus protein is a crystalline globulin, Bawden and Pirie (1940) present chemical analyses of six types of viruses which indicate that they are nucleoproteins. This view is also supported by the work of Rischkaw (1940), McIntosh (1940) and Darányi (1940). It is well established that the virus protein is biochemically and antigenically different from the proteins of the host. In regard to size the viruses appear to exhibit, according to McIntosh, practically a continuous series varying from the smallest 20 m μ to the largest which is 250 m μ . According to Laidlaw (1938) the size

*From “A Theory of Microbic Virulence,” by I. S. Falk in *The Newer Knowledge of Bacteriology and Immunology* edited by E. O. Jordan and L. S. Falk. Reprinted by permission of University of Chicago Press.

varies from 10 $m\mu$ for poliomyelitis virus to 250 $m\mu$ for psittacosis virus. He places the size of vaccine virus at 150 $m\mu$.

New instruments and techniques such as the ultracentrifuge, the electron microscope, and x-ray, are beginning to extend our knowledge concerning the size, morphology, and structure of both viruses and bacteria. By means of the ultracentrifuge, Bauer and Pickels¹ (1940) determined the minimal diameter of yellow fever virus to be 14 $m\mu$ while Gratia² (1940) reports the recovery of polyhedral bodies and of minute granules possessing virulences from serum of diseased silkworms by differential centrifugation.

Borries, Ruska and Ruska (1938) describe a new electron microscope with which they have obtained photographs of several viruses and of bacterial structures previously unrecognized. According to Mudd³ (1941), magnification of 50,000 diameters has been obtained with instruments in the RCA laboratories. He mentions a few limitations of the electron microscope such as the in vacuo requirements, the opacity of specimens of 1 μ or more in thickness, and the possible effect of drying and electron bombardment on the specimen. Bernal (1940) submits x-ray evidence to show that both spherical and rodlike forms exist among the viruses.

There has been much discussion concerning the living or non-living nature of viruses. Rivers (1928) suggests three possibilities as to the nature of viruses. Some may be miniature bacteria; others may be as yet unrecognized forms of life, while a third group may be inanimate transmissible agents of disease.

Green⁴ (1935), Laidlaw (1938) and Gortner (1938) suggest that viruses are living entities that through retrograde changes have lost their enzyme systems and are therefore dependent upon the enzyme system of the host cell. Zinsser and Bayne-Jones (1939) state, however, that they regard the bacterial origin of viruses as highly improbable.

Chemical Composition of Bacteria.—Bacteria have been investigated more extensively from the standpoint of chemical composition and metabolism than any other group of infectious agents. The modern era began with Pasteur's studies in fermentation and his investigation of the germ theory of disease. Koch (1891) obtained from tubercle bacilli a crude group specific protein which

¹Proc. Third International Congress for Microbiology, 1940, p. 287. ²Ibid., p. 288. ³J. Bact. 41: 415, 1941; Ibid. 42: 251, 1941. ⁴Science 82: 443, 1935; Biodynamics No. 39, 1, 1938.

he named tuberculin. Seibert crystallized out the active principle, and she and Long studied its properties. They are discussed in Chapter XXVI.

CARBOHYDRATE.—Zinsser (1923), Heidelberger and Avery (1923), and others have obtained specific carbohydrates from pneumococci and other bacteria. In some instances the exact chemical composition of the polysaccharide has been determined.

PROTEIN.—Furth and Landsteiner (1928) isolated from typhoid bacteria specific protein fractions which they designated as P₁ and P₂. Ando and Ozaki (1930) have shown that scarlet fever streptococci contain nucleoprotein fractions which must be differentiated in the culture from specific soluble toxins secreted by the organisms. Similar studies on *C. diphtheriae* have been carried out by Neill and his colleagues (1930). They have apparently shown that all hypersensitive reactions to products of growth in diphtheria cultures are due to a hypersensitiveness to toxin and that no reactions to nucleoproteins occurred. Extensive chemical studies of many other species of bacteria are being reported in the literature and will be referred to in later chapters.

Effect of Environment on Bacteria.—The cultivation of bacteria in artificial media frequently leads to interesting changes. They may lose their ability to form pigment, to liquefy gelatin, to form capsules or to produce infection as well as many other characteristics. When the environment is modified properly, many of these lost characteristics may be restored.

The loss of characteristics mentioned above has been spoken of frequently as degeneration phenomena. Occasionally an organism acquires a new character and retains it. This is called *mutation*. Neisser (1906) described mutation in a strain of *B. coli*. His observations were soon confirmed by a number of investigators. Barber (1913) and Jordan (1915) have reported mutation arising from the progeny of a single cell.

Bacteria are also able to adapt themselves to growth under many favorable conditions. They may become habituated to growth at temperatures normally inhibitory or to develop in the presence of concentrations of chemical agents such as antiseptics or antisera that are destructive to the original culture.

THEORY OF CONSTANCY OF CHARACTERISTICS.—Cohn (1875) and Koch (1877) are credited with the concept that bacterial char-

acteristics such as morphology, fermentative powers, pigment production, motility, pathogenesis, etc., are constant and inheritable and that all organisms in a colony or pure culture are alike. Opposed to this are the extremely radical views of Nägeli (1877) who held that there is no morphological or physiological constancy.

EVIDENCE OF HETEROGENEITY WITHIN A CULTURE.—It is now quite generally known that among bacteria constituting a pure culture there is definite heterogeneity. The individuals which collectively constitute the culture not only show slight variation in shape, size, and staining reaction but differ in age, physiological stability, pathogenicity and adaptability. Some may be avirulent while others are quite virulent.

FLUCTUATING VARIABILITY.—Heterogeneity in a culture may be due to age, fluctuating variability, heredity, true mutation or to the phenomenon of bacterial dissociation. Fluctuating variability is usually illustrated by variations in size or shape which correspond to height or weight fluctuations observed in the human being. Any characteristic variation that oscillates around an average type is termed "fluctuating variability." Practically all of the phenomena of bacterial variation are discussed by Hadley* (1927) in his excellent monograph on bacterial dissociation. Many kinds of variation are quite evidently examples of either mutation or fluctuating variability, but other variations are difficult to classify.

EARLY THEORIES OF BACTERIAL CHARACTERISTICS.—According to the theory of Cohn (1875), Koeh (1877), Migula (1897), and others that bacterial characteristics are constant, one would expect all colonies obtained by plating a pure culture on agar to resemble not only each other, but also the parent colony. That such is not the case was noted by numerous workers prior to the extensive study of Baerthlein (1918) in colony variation. The latter investigator not only described in detail various types of colonies obtained from each pure culture but he also attempted to correlate colony variation with other characteristics of the organism. Arkwright (1921) is credited by Hadley (1927) with being the first to appreciate the significance of Baerthlein's results.

It would seem, however, that Bordet as early as 1909 described bacterial dissociation into what are now called rough and smooth colonies and found that, while both possessed a common antigenic

*J. Infect. Dis. 40: 1, 1927.

factor, the smooth type contained an additional factor not possessed by the rough variant.

Bordet and Gengou were working with a pure culture of *B. pertussis*. They succeeded in getting it to grow on plain agar and compared the growth and antigenic properties of the organisms grown upon plain and blood agar respectively. The following quotation from Bordet definitely bears out the above conclusions: * "It [*Bacillus pertussis*] develops readily in the medium that is rich in defibrinated blood, as already described by Gengou and myself in our first article on whooping cough. It may be taught to grow on ordinary agar, in which instance it gives a *thick and rather coherent layer*. The two varieties of organisms obtained in this manner, although coming from a single original colony, give rise on immunizing animals to two different sera. We may consider the serum of a rabbit that has been immunized against an organism grown on ordinary agar. It is found that the serum agglutinates these organisms energetically, but has no clumping effect on a culture of whooping cough bacillus grown on the other medium containing defibrinated blood. On the other hand, if we test the serum of a rabbit that has been immunized against the organism grown on blood media, we find that it agglutinates both races of bacteria. A careful study of this phenomenon brings out the fact that two definite agglutinins affecting different antigens are present in different proportions. One of these antigens which is present in large amounts in the organism which has developed on blood is not to be found in the organism grown on agar."

RELATION OF ENVIRONMENT TO SIZE.—In conclusion it would seem advisable to mention a few controversial theories that are being investigated at the present time. It is held by Gotschlich (1927), Kendall (1931), Almquist, Höhnes, Enderlein, Mellon and others that filterable forms of bacteria are demonstrable and that these may give rise to the larger forms. Many of these claims have not been confirmed. A brief but excellent review of the subject is given by Zinsser and Bayne-Jones (1939).

Entirely aside from the question of filtrable forms of bacteria is the effect of the host's tissues upon the size and perhaps morphology of certain organisms. Goodpasture (1937) finds within the walls of the intestinal tract of typhoid patients coming to autopsy

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young plasma cells full of very small rods that stain as if they were viable. These are in contrast to larger rods found in the ordinary phagocytes present in the immediate surroundings. In Goodpasture's opinion these small rods as well as the large ones are typhoid bacteria differing in size due perhaps to their environment. The small ones have made use of young plasma cells as host cells. After liberation from the host cells they developed into the larger rods found within macrophages.

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CHAPTER II

HOST-PARASITE RELATIONSHIP

Introduction.—The environment of man teems with potential pathogenic agents. The soil frequently harbors ova of animal parasites, spores of the gas bacillus and of *Cl. tetani*, as well as vegetative forms of staphylococci and other micro-organisms that survive well outside of the body. It is well known that the surface of the body is commonly contaminated. The skin and mucous membranes harbor streptococci and staphylococci in great numbers, while anaerobes, facultative aerobes, colon bacilli, streptococci, staphylococci and many other kinds of bacteria are present in enormous numbers in the contents of the intestinal tract. Rivers (1928, 1932, 1933) has even found a pathogenic virus on the skin of rabbits. These organisms have very little invasive power and are able to enter the body only through mechanical or chemical injury or as a result of altered physiological integrity of the mucous membranes or skin. Theobald Smith has designated such organisms as "opportunists." They are to be contrasted with the group of infectious agents producing contagious disease and also with the group of agents transmitted by insects.

PORTAL OF ENTRY.—The portal of entry into the body, as well as the route of exit, is quite important in determining infection of the host as well as the danger of spread of the infectious agent. When the portal of entry is through the alimentary canal and the exit is through the feces, as in typhoid fever, bacillary dysentery and asiatic cholera, then *fingers, flies, food, feces* and *fomites* play an important role in disseminating the infectious agents of these respective diseases. In measles and smallpox, where the virus normally enters through the mucous membrane, it appears that *infectious droplets* are chiefly responsible for the spread of both diseases, although fluid from vesicular lesions may be a factor in the spread of smallpox. In diphtheria and scarlet fever, the primary infection occurs in the upper respiratory tract and is spread by direct or indirect contact.

ENDEMIC, EPIDEMICS, PANDEMIC.—When a few cases of an infectious disease occur from year to year in one locality, even though this is quite large, the disease is said to be *endemic* in that region. When more than the usual number of cases develop in a community, the phenomenon is called *epidemic*. The disease may spread to adjacent communities and constitute a large epidemic. When it becomes world-wide in nature, it is called a *pandemic*. A small percentage of the population at large harbors pathogenic organisms known to cause specific infections. These individuals are called *carriers* and are thought to be responsible for endemic disease and for many epidemics. During pandemics, it has been observed that the various diseases spread along the lines of travel, but there seem to be other factors involved that are at present unknown.

INCUBATION PERIOD.—The time interval between infection and the occurrence of symptoms is called the *incubation period*. This is frequently of importance in the spread of infectious diseases since many are more contagious during the latter part of this period when the patient is unaware of the nature of his infection. The duration of this incubation period is quite variable. In diphtheria it is short, being from twenty-four to forty-eight hours, while in typhoid fever, measles, and smallpox, it is commonly two or more weeks.

The converse of infection is freedom from disease. Large areas of population may be free from certain infectious diseases for reasons which may be illustrated as follows:

1. Environmental, Not Involving Immunity

PROTECTION DUE TO LACK OF CONTACT.—European people did not suffer from syphilis prior to the return of Columbus' sailors from the West Indies where syphilis was prevalent among the natives. That this freedom was due to lack of contact rather than to great morality or a high degree of immunity is evident by the virulence of the infections which developed and the high mortality that prevailed after it was introduced. It might be noted here that this is the general experience when a new disease is introduced into a population.

Measles was unknown in the Faroe Islands from 1781 to 1846, when it was introduced from Copenhagen. During the next year over 75 per cent of the population developed the disease.

These two examples indicate that freedom from disease may occur in peoples who are extremely susceptible, merely because they enjoy the freedom from contact. Present quarantine laws have this as one of their objectives.

The same line of reasoning is applied in hospitals where certain types of infections are isolated to prevent spread.

REMOVAL OF PRIMARY HOST.—The attempt of the French to build the Nicaraguan Canal failed because of malaria and yellow fever. The U. S. Government was able to maintain a healthy personnel of labor in the same environment by eliminating mosquitoes, the primary hosts for the infectious agents concerned, rather than by the use of immune individuals in the construction work. Such control may depend upon two possible factors: one is personal prophylaxis against contact with the primary host and the second is the limitation of the habitat of the same. It is obvious that in areas where the primary host cannot survive, a break in the chain of infection will protect the human population. The United States is practically free from bubonic plague. The Public Health Service has combated this disease by measures designed to eradicate the natural host or rodent rather than the insect carrier. During the World War delousing measures were employed extensively in order to prevent the spread of typhus fever and trench fever.

SANITARY MEASURES.—MILLS-REINCKE PHENOMENON. It has been observed that when a large city water supply which has been consistently polluted with sewage is purified by the introduction of filtration and treatment methods, the morbidity and mortality of diseases other than gastrointestinal in origin are noticeably reduced. This is another example of protection by removal of causes by sanitary measures.

It is quite generally conceded that our low morbidity rate for typhoid fever today is due largely to sanitary measures relative to water supplies, milk supplies, oyster industry, and also to food handlers, since only a small percentage of our population has typhoid fever or has been immunized. The corollary to this is the relatively high incidence of typhoid fever among children who are essentially the milk drinkers of the population.

2. Racial Freedom from Disease May Be Due to Customs Rather Than Immunity

EFFECT OF CUSTOMS.—The Hebrew people do not suffer from trichinosis. Since trichina infection is obtained by eating infected or “measly” pork it is obvious that this is not an example of immunity but that freedom is due to racial taboo of pork. Since cases have been reported among Hebrews who violated the taboo accidentally or intentionally, we have no reason to suspect immunity.

3. Environmental Conditions: Absence or Presence of Factors Affecting Immunity Mechanisms

CLIMATE FACTORS.—Dysentery is much less prevalent in the temperate and northern climates than in the tropics. It has been suggested that this illustrates the devitalizing effect of heat. It should not be assumed, however, that heat is the only factor.

Lobar pneumonia and upper respiratory infections are said to be much less prevalent in the tropics than in the colder climates. The inference is made that this may be due to the effect of temperature, humidity, and air currents on the mucous membranes, lowering their resistance. The so-called “fog pneumonia” in England and Belgium reported in 1930 is probably an example of this type. Many occupational diseases come under this group.

4. Resistance and Susceptibility Factors

It seems quite evident from the experimental studies of Topley, Wilson, and Greenwood in England and Webster and others in America that in so far as mouse typhoid is concerned, the addition of fresh mice to an infected population initiates an epidemic.

Webster and Hodes have shown that the mortality, during an epidemic, is conditioned by the number of highly susceptible constituents. When the latter are few in number the deaths will be sporadic; if the number of highly susceptibles is great the mortality will be of epidemic proportions; if the susceptibles are depleted the mortality subsides.

When recruits are added to such surviving populations, made up chiefly of resistants, Webster and Hodes find that mouse typhoid infection spreads to both resistant and susceptible recruits but that the mortality is limited to the susceptible recruits. The innately

resistant recruits remain well unless subjected to conditions that would lower their innate resistance.

There are several reasons why great caution and conservatism should be used in translating the results of experimental epidemiology to human field conditions. These reasons may be summarized as follows:

1. There are many species differences between man and mice. While Webster and Hodes have apparently shown that there is no tendency for susceptible mice to become immunized through herd exposure, their experiments do not prove that susceptible humans will react in a like manner.

2. Man is a heterogeneous mixture as far as breeding is concerned while the mouse research has been done with inbred strains of known pure lines of mice whose susceptibility and resistance could be determined.

3. The experimental epidemics in mice were carried out under controlled conditions as to recruits, diet, temperature, humidity, etc. This is in great contrast to epidemic disease in man. Such epidemics occur spontaneously in a mixed population that exhibits great variation in age, history of previous disease, diet, health habits, natural fitness, occupation, previous immunization, etc. The source of the infection, the number of susceptibles, resistants, and carriers, as well as the number and condition of new recruits, are usually unknown.

4. In a mouse population there is no conscious effort on the part of individuals to protect themselves and others against infection such as occurs during epidemics in man. Since there is in every human population great variation in education, knowledge, wisdom, and prejudice, it is not surprising that co-operation of the public in applying what appears to be intelligent methods of control is not accomplished.

In spite of these reasons for caution it would seem that experimental epidemiology is making major contributions to our knowledge of infection and host-resistance.

In man the carrier state (both temporary and chronic) presents an interesting host-parasite relationship that in many cases develops into a public health problem. There is a great deal of data bearing upon the incidence of typhoid, diphtheria, meningococcus and pneumococcus carriers in a population but relatively little

information concerning the virulence of the organisms isolated from carriers. Hadfield and Garrod (1938) review the studies in the distribution of pneumococcus types in normal individuals reported by Webster and Hughes (1931), Gundel and Linden (1931). They say that the evidence indicates that the less virulent types attack the respiratory tract, set up mild infection, establish themselves and remain in intimate relationship with the host for long periods of time. This is in contrast with the virulent types which must either produce an acute infection or be destroyed.

Theobald Smith taught long ago that lymph glands are filters. Failure to realize that bacteria thus filtered out may survive for long periods of time within the lymph glands is probably the reason so many different kinds of bacteria have been described as the cause of Hodgkin's disease. The phenomenon of phagocytosis observed in various diseases is regarded by Smith as an expression of an affinity possessed by certain infectious agents and cell types of the host for each other. This subject is discussed more fully in Chapters IV, V, and XXVII.

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CHAPTER III

INFLAMMATION AND LEUCOCYTE RESPONSE

Introduction.—In regard to the phenomena of infection and its sequelae the older clinicians carefully describe inflammation as the reaction of the tissues to injury. Virchow and succeeding pathologists have reported upon the microscopical changes which occur. Experimental hematologists such as Ehrlich, Sabin, Doan and Cunningham, Maximow, Downey, Bunting, Pappenheim, and others have introduced methods of staining, identifying and classifying the various types of cells observed in the blood, inflammatory exudates, and lesions and have attempted to determine their origin (Plates I and II). Arneth, Schilling, and more recently Haden have suggested that in acute infections the activity of the bone marrow (Plate I, 1) is reflected by the relative number of young forms of neutrophils in the peripheral circulation. They believe that in acute infections a prognosis can be based upon indices or hemograms giving this information and accordingly suggest formulae for such indices and hemograms.

Acute Inflammation. (1) **LOBAR PNEUMONIA.** In acute lobar pneumonia, which is usually caused by the pneumococcus, there is an acute inflammation of at least one lobe of the lungs accompanied by systemic disturbance. It is ushered in with a chill, fever, leucocytosis, rapid pulse, and rapid respiration. Obviously there is a disturbance of respiratory function. The inflammatory process in the lung is characterized by several stages.

Like all acute inflammatory processes the first stage is one of hyperemia or increase in blood supply to the infected lobe. In pneumonia this is called the *stage of engorgement*. During this stage there results a change in permeability of the vessel walls, a slowing down of the rate of blood flow and a margination of leucocytes. This is also common to all inflammatory processes. Leucocytes are attracted to the infected lungs. Fluid, leucocytes, and even some red cells pass through the vessel walls into the alveoli.

The second stage is ushered in by the consolidation of the lung due to the clotting, so to speak, of the inflammatory exudate. This

is called the *stage of red hepatization* or liverlike appearance and consistency of the lung. The microscopic picture at this stage would show the alveolar walls thickened, the capillaries distended, the alveoli filled with coagulated albuminous fluid, leucocytes, mononuclear cells, bacteria, some red cells, and a great deal of fibrin.

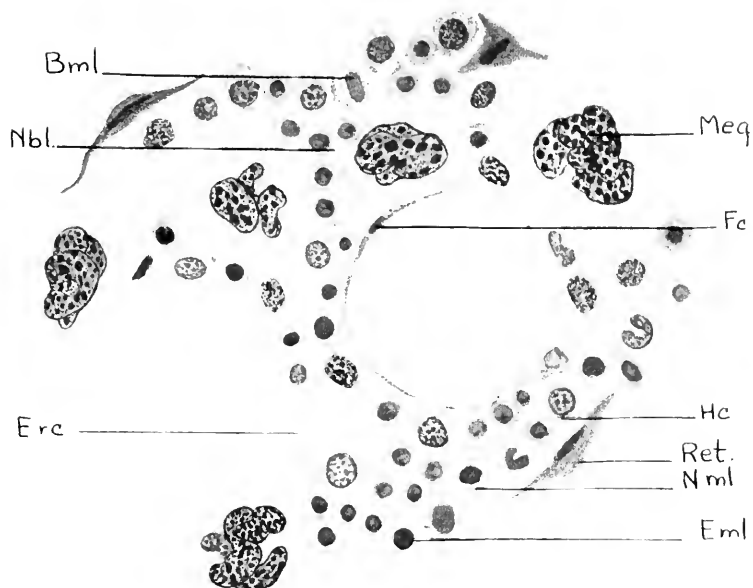
The next stage is one described as "*gray hepatization.*" The red cells either lose their hemoglobin or are destroyed while the lung remains otherwise the same. The *last stage* is that of *resolution*. Proteolytic enzymes of leucocytic origin (from leucocytes that are destroyed) liquefy the coagulated proteins and the products are eliminated in the sputum and urine and the lung returns to normal. The bacteria have been phagocytized by neutrophils, free mononuclear and fixed mononuclear cells. The phenomenon of crisis which is generally regarded as peculiar to lobar pneumonia in man has been experimentally duplicated by Goodner in his "*dermal pneumonia*" (intra-dermal infection) in rabbits.

(2) **FURUNCLE.**—Another interesting example of acute pyogenic infection in man is the furuncle (boil) due to the staphylococcus. Infection usually occurs around a hair follicle. Here one observes hyperemia, exudation, the accumulation of fluid, leucocytes, red cells, fibrinogen, and bacteria in the area of inflammation. Coagulation of the protein occurs. There is a piling up of neutrophils and mononuclear cells at the outer margin of the spherical zone of coagulation. The small blood vessels and lymphatics are injured and become plugged with thrombi and hyaline plugs, respectively, which tend to prevent the spread of the infection.

In the furuncle the coagulated mass of cells dies. Here again proteolytic enzymes of leucocytic origin begin to liquefy the coagulated mass which in this case contains dead tissue. Liquefaction begins at the outer margin and progresses toward the center. The liquefied tissue debris, dead and living leucocytes, bacteria, fibrin, etc., constitutes the *pus* that one sees while the unliquefied central portion is the "*core*" of the boil. The cavity thus formed is lined with a membranous-like material made up of fibrin, various types of white cells, tissue cells, etc., and is called the *pyogenic membrane*. It, with its phagocytes and the hyaline and fibrinous plugs in the lymphatics and blood vessels, acts as a protective barrier to the spread of infection.

PLATE I.—BLOOD CELLS (FIXED AND STAINED PREPARATIONS)

1. Section of bone marrow—guinea pig.
Bml—Basophilic myelocyte.
Nbl—Erythroblast.
Ere—Erythrocyte.
Meg—Giant cell.
Fc—Fat cell.
He—Histiocyte.
Ret—Reticular cell.
Nml—Neutrophilic myelocyte.
Eml—Eosinophilic myelocyte.
2. Erythrocyte.
3. Blood platelets.
4. Large lymphocyte.
5. Small lymphocyte.
6. Monocyte.
7. Neutrophile (mature).
8. Basophile.
9. Eosinophile.
10. Monocyte (showing azurophilic granules).



1

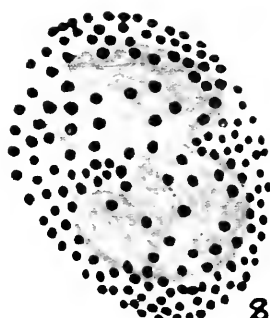
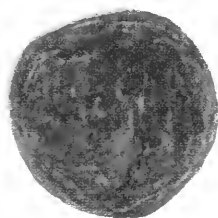
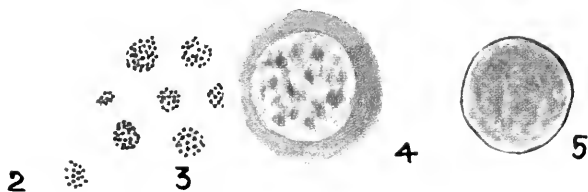


PLATE I.

(See explanation on opposite page.)

Menkin, Opie, and others have shown that when one injects dyes, proteins, etc., either locally or intravenously the material is picked up and fixed by areas of inflammation. The final process in recovery is the replacement of dead tissue by connective tissue which through changes in the fibers and shrinkage forms scar tissue. It is obvious that if scar tissue forms in the walls of the esophagus, intestines, ureters or urethra, the lumen will be narrowed by a stricture and functional impairment will result. The numerous investigations relative to the cellular (phagocytic) defense of the body against streptococcus, staphylococcus, and other infectious agents which have been cited all point to the great importance of fixed and free mononuclear cells rather than the neutrophiles in combating the infection.

Chronic Inflammation.—FORMATION OF A TUBERCLE.—In tubercular and many chronic inflammations it has been noted that there may be very little hyperemia, local increase in temperature or exudation of fluid. Neutrophiles may appear early, but they are soon supplanted by mononuclear cells. Pus may be formed as in the "cold abscesses" of tuberculosis with little systemic disturbance.

Maximow, as well as Sabin, Cunningham, and Doan, has given excellent descriptions of the formation of the tubercle under experimental conditions. When tubercle bacilli set up a primary infection in tissue, there is an early appearance of neutrophiles, mononuclear cells, a few red cells, and some fluid. The first inflammatory reaction subsides and the exudative process disappears. The mononuclear cells then increase in number and collect in a mass around the tubercle bacilli. The mononuclear cells, according to Maximow, gradually undergo transformation. Their cytoplasm becomes more abundant and acquires the capacity to stain with acid dyes. Near the center of the mass one or more "giant cells" appear. These are the giant cells of Langhans. They are described as "protoplasmic masses with the nuclei arranged in a peripheral ring or in an equatorial band." It is Maximow's opinion that they are formed by the fusion of the large mononuclear or epithelioid cells and that mitosis of the nuclei follows.

Sabin et al. classify the mononuclear cells forming the tubercle as monocytes. They did not observe fusion of monocytes to form

PLATE II.—CELLS IN PERITONEAL EXUDATE OF WHITE RAT. SUPRA-VITAL
PREPARATION USING NEUTRAL RED AND JANUS GREEN

1. Lymphocyte showing both neutral red granules and mitochondria.
2. Lymphocyte showing only mitochondria.
3. Clasmatoocyte showing vacuoles of neutral red and mitochondria.
4. Monocyte showing the rosette and mitochondria.
5. Clasmatoocyte which has phagocytized a neutrophile (note that the nucleus of the neutrophile, being dead, stains with neutral red).



1



2



4

3

5

R Blood

PLATE II.

(See explanation on opposite page.)

giant cells but think they are formed by mitosis and increase in cytoplasm. There is no blood supply extending into the mass or tubercle.

A mantle of granulation tissue due to connective tissue proliferation forms about the mass of mononuclear cells forming the tubercle. Small mononuclear cells resembling lymphocytes tend to collect in and about the outer elements of the mantle of granulation tissue.

TUBERCLE PHOSPHATID AND CELL STIMULATION.—Sabin and Doan (1927) report that Anderson's phosphatid fraction of the tubercle bacillus will cause "decided proliferation of monocytes, epithelioid cells, and giant cells leading to a massive formation of tubercular tissue."

VARIETIES OF THE TUBERCLE BACILLUS.—Winn and Petroff* (1932) have obtained four interesting variants which they call S, F.S., R and Ch. dissociated from an avian tubercle bacillus A¹. They state that these variants show different physical and chemical properties and elicit different tissue responses in the host. They summarize their results as follows:

"The leucocyte response in S and F.S. is of an acute type while that produced by R and Ch. variants is indicative of a chronic, healing tuberculosis.

"The tubercle formed by S is of an acute toxic type, the F. S. more of a foreign body type and that of R and Ch. relatively benign.

"The S variant is by far the most virulent and is closely followed by the F. S. type. The R and Ch. variants are comparatively avirulent."

DEVELOPMENT OF HYPERSENSITIVENESS.—Primary infection with the tubercle bacillus renders the animal hypersensitive to tuberculin. This state of hypersensitiveness is responsible for the violent inflammatory reaction around new and latent foci of infection which develops when reinfection with tubercle bacilli occurs or when a normally nontoxic dose of tuberculin is injected into an infected animal. It is responsible also for many systemic symptoms which are known to be pathognomonic of tuberculosis.

Tissue Response to Viruses.—In regard to virus disease one is dealing with infectious agents that enter the cells of the host and

*Winn, W. A., and Petroff, S. A.: J. Exper. Med. 57: 239, 1932.

bring about certain changes which usually result in a secondary inflammation accompanied by mononuclear cell infiltration. According to Rivers (1933) if the virus does not act rapidly and explosively and the host cells are capable of multiplication, the primary effect of infections is stimulation leading to cellular hyperplasia. Following the latter there is usually necrosis and then a secondary inflammation. Rivers states that the balance between stimulative and destructive tendencies of the virus is the determining factor between predominance of hyperplasia and necrosis respectively. He cites smallpox lesions as examples of stimulation followed by necrosis and liquefaction of cells, i.e., there is first a hyperplasia of cells followed by vesicle formation. As examples showing an overgrowth of tissue as the prominent feature he cites warts and certain tumors. When the infected cell "is incapable of dividing and multiplying as is the case of nerve cells, then the primary pathological changes are necrobiosis and lysis of cells or the appearance within the cellular elements of inclusion bodies." This latter phenomenon is observed in rabies.

Leucocyte Count.—It is an interesting physiological fact that in a normal individual the red cells, white cells, and the platelets are maintained at a fairly constant level. Walters (1934) has shown that exercise and rest affect the concentration of red cells in the venous circulation. Doan (1927) and others have observed a rhythmical variation in the number of white cells. Mueller has shown that the vegetative nervous system exercises some control over the number and distribution of white cells. It is also of interest to note that differential counts indicate that there is to a great extent a constant percentage of each type of white cell in the peripheral circulation. Under normal conditions there are, on an average, approximately 5,000,000 red cells and 7,500 white cells per cubic millimeter of capillary blood.

There are a few infectious diseases characterized by a leucopenia (low white count). Among these diseases are measles, malaria, influenza, typhoid fever, and tuberculosis. The differential count of a normal blood shows that there are approximately 60 to 70 per cent neutrophils, 2 to 8 per cent monocytes, 25 to 30 per cent lymphocytes (large, intermediate and small), 1 to 3 per cent eosinophiles, and from $\frac{1}{2}$ to 1 per cent basophiles. In the five infec-

tious diseases just mentioned there are a relative increase in mononuclear cells and a relative decrease in granulocytes.

In lobar pneumonia, meningitis, and severe septic infection the white count is usually 20,000 to 30,000 with a high percentage (over 90 per cent) of neutrophiles. Occasionally one finds a low total white count with a high percentage of neutrophiles in malignant septicemias. In appendicitis great variations are observed. One usually thinks that a patient with a "pus appendix" will have a white count of between 16,000 and 20,000 with 90 to 98 per cent neutrophiles. On the other hand, cases of catarrhal appendicitis usually show white count under 15,000 per cubic millimeter and from 80 to 85 per cent neutrophiles. Exceptions are frequently encountered, hence the surgeon must always regard the laboratory findings as of less importance than his clinical findings and the condition of the patient.

The student should become familiar with other conditions (besides infection) that affect the white count. There is usually a leucocytosis following hemorrhage of any consequence; and an increase in leucocytes is observed in pregnancy, during labor, and also during digestion and after cold baths. A leucocytosis is frequently observed when the patient is moribund and is probably due to a terminal infection.

In acute infections, Sondern suggests that the percentage of neutrophiles indicates the degree of toxic absorption while the number of leucocytes per cubic millimeter of capillary blood reflects the power of resistance of the patient. Wilson (1919) has devised a formula to express this information in numerical terms. The student should consult his original papers for a more extensive discussion of the subject. Todd and Sanford (1931, p. 293) incorporate Wilson's formula in a short discussion of his work.

ARNETH AND SCHILLING COUNTS.—In an attempt to obtain additional information that might indicate the severity of an infection and perhaps whether the prognosis is good or bad, Arneth and more recently Schilling (1929) and Haden have suggested the use of indices and hemograms based upon the relative number of young and old neutrophils in the peripheral circulation at different stages of infection.

According to Schilling the percentage of different kinds of white cells in normal blood is:

Basophiles	0.5 per cent
Eosinophiles	2-4.0 per cent
Myelocytes	0.0 per cent
Juveniles (Metamyelocytes)	0.0 per cent
Stabs	3-5.0 per cent
Segmented	51-67.0 per cent
Lymphocytes	21-35.0 per cent
Mononuclears	4-8.0 per cent

The myelocytes and metamyelocytes (juveniles) are bone marrow cells and give rise to the granulocytes of the peripheral circulation. Gradwohl has described the myelocytes, juveniles and stabs as follows:

“**NEUTROPHILIC MYELOCYTES.**—The cytoplasm of neutrophilic myelocytes varies from very pale blue in the younger stages to pink in the older forms. The nucleus varies in shape; round, kidney-shaped, or oval. It may or may not contain one or more nucleoli, and is trabeculated. In leucemia the granules of the myelocytes are very delicate and difficult to stain. They are purple and pinpoint in size. They are seen usually only here and there; at times they may be entirely absent. In the blood stream in severe infections, myelocytes usually have a pale blue cytoplasm filled with coarse neutrophilic (purple) toxic granulation. The myelocyte of leucemia has a fairly even periphery, while that of infections is fragile, and consequently irregular.

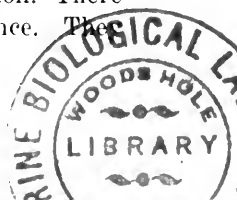
“**JUVENILE NEUTROPHILES.**—Juvenile neutrophiles are found normally in the circulating blood in the percentage of 0 to 1 per 100 leucocytes. They are slightly larger than the mature neutrophils. The cytoplasm varies from a bluish pink to a definite pink. It is much wider than that of the myelocytes. The nucleus is sausage-shaped to almost bean-shaped. It does not stain intensely, and shows little or no chromatin structure, although it is definitely divided into fields. It often contains one or more nucleoli, usually situated in the end bulbs, which are definitely protruding structures. The granules are sometimes definite and sometimes very fine. When they are coarse, they are strikingly responsive to stain; when fine, they stain with difficulty. The same variations in staining and structure may be noted in leucemia and infections as is seen in the myelocytes. They are differentiated from ‘stab’ cells

by the pale nucleus without chromatin, the nucleoli and the width of the nucleus. 'Stab' cells are older, and consequently stain more deeply, have no nucleoli, show dense chromatin structure, and have a fairly narrow nucleus. When any *reasonable* doubt exists as to whether a cell has reached the 'stab' stage or is still a juvenile, Schilling suggests that it be called a 'stab.'

" 'STAB' OR BAND OR ROD NUCLEAR NEUTROPHILIC CELLS.—The term 'stab' is a German word which refers to the ability of the rod-shaped nucleus to become bent or twisted. Since there is no handy English equivalent, the German term has been retained. Numerous synonyms are found in the literature; such as, 'staff cells,' 'rod nuclears,' 'nonfilamentous forms,' 'band cells,' etc. 'Stab' cells are neutrophilic cells and are found in the normal circulating blood in the percentage of 3 to 5. The cytoplasm is pink or lavender. The nucleus is a definite flexible rod, which may be bent in smearing, so that it takes the form of the letters S, T, U, V, or W. It does not show segmentation, although degeneration forms in which the nucleus is vacuolated or fringed may occur. Any cell which shows even slight segmentation is not considered a 'stab' cell. The nucleus contains definite chromatin structure, which must not be mistaken for nucleoli. It stains more intensely than the nucleus of the juvenile cell, and is slightly narrower. It does not contain nucleoli.

"The granules are usually distinct, very fine, purple-staining stippling, distributed less regularly than the granules of the eosinophiles, but more regularly than those of the basophiles. Under conditions of severe infections, they are very coarse and clumped. If there is any *reasonable* doubt as to whether a cell is a 'stab' or a segmented form, Schilling suggests that it be called a segmented cell. The nucleus of the stab may undergo changes due to mechanical pressure in smearing. Unusual forms are to be looked for and recognized.

"It is important to remember, too, that there are *degenerative* forms of the stab cells. The degenerative forms show small band forms of nuclei, irregular, hyperchromatic, pyknotic, and structureless. They are easily broken up in making the preparation. There is tendency toward vacuolization and diminished resistance. They are a result of a toxic element or infection."



The segmented forms constitute the major portion of the neutrophils. The cytoplasm is mature and filled with neutrophilic granules. The nucleus consists of two to five unequal segments united by fine threads. A Schilling hemogram follows:

HEMOGRAM*

	LEUCOCYTE COUNT	BASOPHILES ¹	EOSINOPHILES ¹	MYELOCYTES ¹	JUVENILES (METAMYELOCYTES OF PAPENHEIM) ¹	STAB NUCLEARS (NEUTROPHILES WITH SINGLE LOBE) ¹	SEGMENT NUCLEARS ¹	LYMPHOCYTES ¹	MONOCYTES ¹	THICK DROP OBSERVATIONS AND REMARKS
Normal limits	5,000 to 8,000	0 to 1	2 to 4	0	0 to 1	3 to 5	51 to 67	21 to 35	4 to 8	

*After Schilling: The Blood Picture, The C. V. Mosby Co., p. 148.

¹Per cent.

From an inspection of the differential count recorded in the above hemogram it is obvious that the young forms of neutrophils (stab forms) and their precursors are recorded to the left of the line separating the stab forms from the older or segmented neutrophils. For various reasons Schilling has found it expedient to place the basophiles and eosinophiles on the left and the lymphocytes and monocytes on the right.

When a blood count is made on a patient suffering from an acute pyogenic infection, the percentages of the various types of cells are recorded in this form of hemogram. When the sum of the percentages to the left or right of the dividing line between the stabs and segmented nuclears shows an increase above normal, it is recorded as a shift to the left or right as the case may be. In acute sepsis, appendicitis, etc., there is a high total white count with a regenerative shift to the left. This is due to the appearance of myelocytes and juvenile forms in the peripheral circulation and also to an increase in stab forms.

Schilling thinks that repeated counts are of great value in making a prognosis. A continued or increased shift to the left is regarded as unfavorable, while a shift to right is indicative of a

favorable prognosis. In two of the diseases, e.g., tuberculosis and typhoid fever, in which a leucopenia is commonly encountered, one may observe a degenerative shift to the left. Juvenile forms and myelocytes do not appear in the peripheral circulation, but there is an increase in the stab forms. Schilling interprets the blood picture during the course of an acute infection according to three phases:

Phase I. The neutrophile battle phase.

Leucocyte count	B	E	M	J	St.	S	L.	Mono.
Count increased	0	0	0	16	8	55.5	18	2.5

This phase characterized by neutrophilia with a severe regenerative nuclear shift. Lymphopenia, monopenia, and eosinopenia occur at the peak of the infection.

Phase II. The phase of monocyte defense.

Leucocyte count	B	E	M	J	St.	S	L.	Mono.
High normal	0	2	0	0	7.5	58.5	15	17

Here one knows from the receding neutrophilia and the shift to the right, from the reappearing eosinophiles, the rising number of lymphocytes and the high number of monocytes that the critical terminal period of the infection has arrived when the patient puts up an energetic defense.

Phase III. The phase of lymphocyte cure.

Leucocyte count	B	E	M	J	St.	S	L.	Mono.
High normal	0	7	0	0	4	33.5	42.5	13

This stage is characterized by lymphocytosis, eosinophilia with no neutrophilic shift.

Schilling says that the persistence of a high neutrophilic shift and high monocytosis with few eosinophiles indicates a condition of chronic infection.

LEUCOPENIA.*—An excellent discussion of the factors involved in leucopenia is given by Lawrence (1941). He lists the following five major mechanisms which may operate in the production of this condition:

1. Diminished production of leucocytes due to (1) simple inhibition, (2) arrested maturation, (3) bone marrow aplasia and (4) infiltration of foreign cells into the bone marrow.

*A recent paper of interest is by Olitzki, L., Avinery, S. H., and Bendersky, J.: The Leucopenic Action of Different Microorganisms and the Antileucopenic Immunity, *J. Immunol.* **41**: 361, 1941.

2. Excessive elimination of leucocytes through such normal channels as the lungs, gastrointestinal tract, liver, or spleen or under pathological conditions when large numbers of cells are poured into an infected area such as empyema.

3. Excessive destruction of white cells due either to abnormality of the white cells or to leucotoxic substances in the blood.

4. Redistribution of leucocytes in the vascular channels such as occurs from the intravenous injection of hydrophilic colloids. The peripheral leucopenia apparently results from a mobilization of the granulocytes in the internal organs.

5. Redistribution of leucocytes in the body as a whole. He cites leucopenic phases of leucemia as an example of the breakdown of the mechanism responsible for the distribution of leucocytes between the tissues and vascular channels.

In discussing Lawrence's report Haden (1941) suggests that since leucopenia, from the clinical point of view, concerns the polymorphonuclear cells almost entirely, the term granulopenia seems preferable. Haden also points out that the life of a white cell is probably not over four or five days and that therefore the normal demand for replacement is probably 5 to 10 billion new cells per day. In his opinion almost all clinical cases showing granulopenia belong to Lawrence's first group.

LEUCOCYTOSIS AND INCREASED CAPILLARY PERMEABILITY.—Any consideration of leucocytosis associated with inflammation should not only consider the phenomenon of increase in the number and kind of leucocytes but should also consider the phenomenon of chemical attraction of the neutrophils to the point of infection (positive chemotaxis) and their passage through the vessel walls respectively. According to Menkin (1940) numerous investigators have found that the injection of nucleic acid into animals produces an initial leucopenia followed within a few hours by a leucocytosis. Splenectomy apparently favors the production of a leucocytosis without the initial leucopenic phase.

It is reported that the Arneth or Schilling count is deflected (shifted) by the administration of irradiated ergosterol, gelatin, trypsin, nucleic acid, thyroxin, and colechicine. Moon* (1938) regards histamine or an H-substance as of importance in the production of a leucocytosis associated with inflammation. He found that the intravenous injection of 1 or 2 mg. of histamine into cats pro-

*Moon, V. H.: Pathology and Mechanism of Anaphylaxis, *Ann. Int. Med.* 12: 205, 1938.

duced a leucocytosis. Menkin (1940), however, reports that the intravenous injection of similar or slightly higher amounts into dogs failed to alter appreciably the leucocyte count.

In connection with his studies on inflammation, Menkin (1936, 1938, 1939, 1940) has investigated experimentally the various phenomena associated with phagocytosis mentioned earlier in this section. His results may be summarized as follows:

1. He has obtained a crystalline nitrogenous substance from inflammatory exudates which increases capillary permeability and causes the migration of leucocytes through the vessel walls into the tissues. While it exhibits chemotaxic properties it will *not* produce a leucocytosis in either the dog or the rabbit. This substance is called "*leucotaxine*."

2. He has found a *leucocytosis-promoting* factor in inflammatory exudates that causes a rise in the level of leucocytes in dogs. It stimulates the bone marrow causing a "shift to the left" in the hemogram. This factor is thermolabile (inactivated at 60° C.) and there is some evidence to indicate its being either a globulin or associated with the globulin fraction.

3. Menkin reports that histamine, adenosine, blood serum, sterile broth and cultures of *Staphylococcus aureus* are all *ineffective* in causing an increase of the level of leucocytes in the circulation.

4. The leucocyte-promoting factor causes a prompt leucocytosis whereas nucleic acid produces a delayed reaction.

It is suggested that the student read a few of the papers bearing upon the Arneth and Schilling counts which are included in the list of supplementary references. From a survey of the literature and some personal experience, one feels warranted in suggesting that the student weigh carefully the claims of those authors who feel that any method of classification of white cells is infallible in prognosis. While the Schilling count is often of value, it is also at times misleading. It is well to remember that even accurate laboratory findings must be interpreted by a trained, intelligent individual with considerable clinical experience.

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CHAPTER IV

ANATOMICAL AND PHYSIOLOGICAL FACTORS IN INFECTION AND RESISTANCE OF THE INDIVIDUAL

When the opportunity occurs for an infectious agent to come into contact with the animal body, it is said that the animal is *exposed* to infection. The portal of entry, dosage, and virulence of the pathogen, as well as the anatomical and physiological condition of the host are factors determining whether entrance is gained to the body and whether disease results.

The significance of many of these factors has been described in the preceding pages. Attention has been called to the presence of myriads of potentially pathogenic micro-organisms present on the body surfaces awaiting an opportunity to invade the tissues.

The first line of defense against invasion of the tissues is the epithelial coverings of the body and the various secretions which bathe them. The second line of defense consists of cells of the reticulo-endothelial system present in the skin or mucous membranes and in the lymph glands which drain these areas. When one considers the central nervous system, he finds that the choroid plexus seems to act as a barrier between the general circulation and the circulation of the brain and cord.

The skin, with its outer insoluble, keratinized epidermis, its multiple layers of stratified squamous epithelium, and rich capillary blood supply, offers a substantial barrier to bacterial invasion. Williams (1941) says that the nature and low pH of the outer layer discourage growth and invasion of bacteria. In his opinion the resurfacing mechanism of the body is important in defense.

While the *mucous membrane* of the buccal cavity, as well as those of the nose and throat are known to harbor a rich bacterial flora, they are protected to a certain extent from contact with bacteria by a film of mucus. Secretions of the submaxillary and parotid glands enter the buccal cavity through their respective ducts while secretions from the bronchi and trachea are carried upward to the mouth by cilia of the trachea. The physiologic vigor and integrity of the mucous membrane are dependent, to a large

extent, upon the rich blood and nerve supply with which it is endowed. The coughing reflex, depending as it does upon a nervous mechanism, aids at times in the elimination of contaminated material.

Bloomfield* (1922) suggests that there are six factors of possible importance in the elimination of bacteria from the respiratory tract. These factors he enumerates as follows: "(a) anatomic conditions; (b) the flushing mechanisms; (c) bactericidal action of the secretions; (d) reactions of secretions; (e) the antagonistic action of the indigenous flora toward invaders; and (f) phagocytosis." He calls attention to the importance of the swallowing reflex as a protective mechanism and cites experiments showing that the tonsils are subjected to very little exposure to bacteria contained in the food or fluids entering the mouth.

On the other hand, air currents entering the nose impinge immediately upon the mucous membranes. A large number of bacteria present in dust particles or in infectious droplets are arrested very near the anterior nares. Those that succeed in passing this barrier are caught in the mucous film covering the nasal membranes and swept toward the pharynx and esophagus by the cilia of the epithelium.

Located in the upper respiratory tract is also the *tonsillar ring* consisting of the faucial tonsils, lingual tonsils, and adenoid tissue. The latter is located in the dome of the nasopharynx and is structurally lymphoid tissue. Since the tonsils contain many crypts in which bacteria may find lodgment, it is not surprising that they are frequently the site of acute or chronic inflammation. Like all lymphoid tissue they contain along with lymphocytes other white cells that are phagocytic.

Directly associated with the upper respiratory tract and communicating with it are certain other *structures*, for the most part lined with the same kind of pseudostratified epithelium as that covering the nose. These structures include the *ethmoid, sphenoid, frontal, and maxillary sinuses*, as well as the *middle ear and conjunctiva*. The lacrimal secretions are mildly germicidal and also aid in the mechanical removal of bacteria.

Should bacteria enter the sinuses they would become entangled in the mucous film which is propelled to the opening of the ducts

*Bloomfield, A. L.: Am. J. M. Sc. 164: 854, 1922.

by cilia of the epithelium. Undoubtedly the mucous secretions, drainage, rich blood supply, and type of epithelium together with certain phagocytic cells constitute the defensive mechanism of this region. It should be apparent that any interference with the drainage of the sinuses would impair an important defensive mechanism. The paralysis of the cilia by improper treatment or the occlusion of the ducts by swelling of the nasal mucous membrane or occlusion by a deviated nasal septum can impair sinus drainage.

This region of the paranasal sinuses and upper lip is called the "*danger area*" because the veins draining into the blood sinuses of the head are for the most part without valves and the spread of infection to the blood sinuses would mean the development of meningitis. The proximity of the middle ear to both the mastoid and the lateral sinuses is exceedingly important in the spread of infections.

Since the roots of the upper teeth (Fig. 1) are in close proximity to the floors of the maxillary sinuses, apical infections may lead to sinus involvement.

Mechanisms of Infections.—Just how infectious agents are able to *penetrate* surface barriers is not always clear. Several possibilities are usually suggested as explanations. It is obvious that chemical injury or trauma (mechanical injury) will permit entrance. Micro-organisms might be taken up by leucocytes present on the surface and these leucocytes might enter the tissues by diapedesis (somewhat as a drop of mercury passes through gelatin). Yoffey and Sullivan (1939) describe the fixation of vaccine virus by the lymphocytes and the spread of the virus via the cervical lymph ducts to the blood stream.

If bacteria are able to kill the leucocyte after entering or they escape through disengagement, it would seem logical to assume that the bacteria might be able to grow and develop once they get established. A third possibility is that bacteria may colonize upon the surface and produce substances that kill the epithelial cells adjacent and thus directly find themselves in contact with underlying structures. Such a condition can be readily visualized as occurring when pathogenic organisms are sealed off within a tonsillar crypt or a hair follicle. Undoubtedly the bacterial protein or the products of bacterial growth frequently affect the permeability of the cell membrane.

Mudd (1924) suggests that perhaps an electrocapillary mechanism plays a role in the penetration of the epithelium by virulent bacteria. That physiological factors of the host are of paramount importance in infectious processes is evident from the fact that in insulin deficiency, such as occurs in diabetes, a state of acidosis may occur and coincident with it the surface resistance against

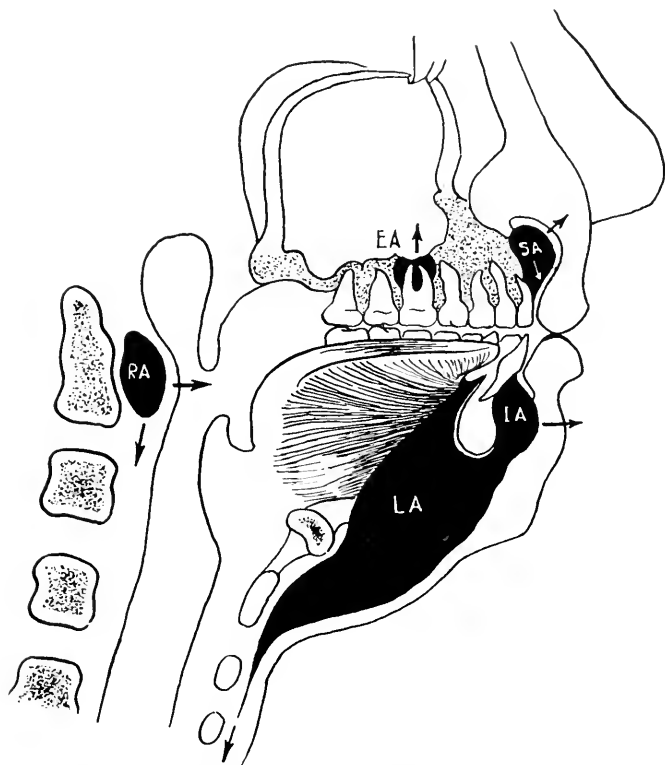


Fig. 1.—Sagittal section of head to show spread of suppuration from infected teeth; and also location of retropharyngeal abscesses. (After Eisendrath, *Surgical Diagnosis*, p. 128, by permission of W. B. Saunders Company.)

SA, Subperiosteal abscess of upper jaw. IA, Subperiosteal abscess of lower jaw. LA, Infection in submaxillary subcutaneous tissue. EA, Infection around roots of bicuspid and molar teeth. RA, Retropharyngeal abscesses.

“opportunists” is lowered as evidenced by the common occurrence of sinus infection and skin infections (boils and carbuncles) in these patients. Likewise frequent attacks of rhinitis are common occurrences in patients suffering from tuberculosis or other debilitating diseases.

ESTABLISHMENT WITHIN THE TISSUES.—After the invasion of the body surface (in trauma), it is necessary for the organisms to establish themselves preparatory to dissemination elsewhere in the body. Just how the pathogen establishes itself is unknown. Wherry (1927) thinks that the bacteria or their products of growth cause hydration of the tissue and that this enables them to grow and multiply. He calls attention to the fact that there are found in filtrates of certain bacteria (e.g., *C. diphtheriae*, *Str. scarlatinae*, *Str. erysipilatis*, *Cl. welchii*, etc.) substances which produce local congestion and edema (water in the tissues). He suggests that the edematogenic substances may be amines. His experimental approach is exceedingly interesting and suggests that in the hydration of proteins the bacteria may have a mechanism that enables them to grow and multiply. That the mere production of edema is not always sufficient is suggested by the lack of serious infection in many cases of extreme edema of the lower extremities in women where the edema is caused by the mechanical pressure of a large ovarian cyst in the abdomen. This does not invalidate Wherry's conclusions that the hydration of tissues by infectious agents aids in their growth and development.

There are certain kinds of infectious agents such as the viruses, rickettesia, and *B. leprae* that are intracellular parasites and require the presence of susceptible cells in which to multiply. Theobald Smith (1933), and Goodpasture and Anderson (1937) have emphasized the importance of host-cell-parasite relationship in certain diseases. In their studies of experimental infections of the chorio-allantoic membrane of chick embryos, Goodpasture and Anderson (1937) found that many pathogenic bacteria find either epithelial or mesodermal cells are both favorable and perhaps necessary media for the invasions of the living host. Their work will be discussed more extensively in Chapter VII.

Routes of Dissemination.—After infection is established there are certain avenues of spread or routes of dissemination of the infectious agent to be considered. The four avenues commonly mentioned are *surface spread*, dissemination by way of the lymphatics (*lymphogenous*), blood stream (*hematogenous*) and *direct extension* to adjacent tissues. The first of these can be illustrated by infection of the nasopharynx. The infection may extend downward over the mucous membrane to the tonsil, trachea, and lungs

or be forced through the Eustachian tube (auditory canal) to the middle ear or to any of the adjacent structures covered by extension of the mucous membrane of the nose and throat.

LYMPHOGENOUS EXTENSION.—All of the structures just mentioned, as well as all of the tissues of the body, are extensively supplied with *lymphatics* which drain into regional lymph glands (see Figs. 2, 3, and 4). The spread of vaccine virus by the lymphatics described by Yoffey and Sullivan (1939) has been men-

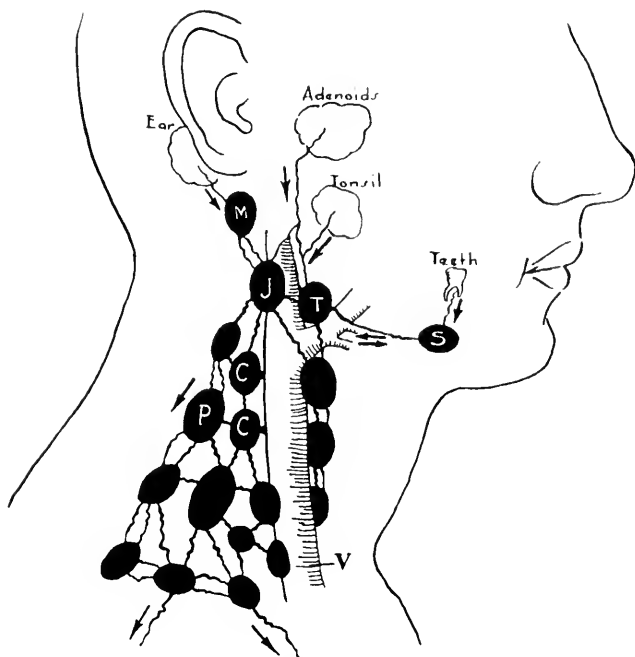


Fig. 2.—Portals of infection and the most frequent nodes involved in tuberculosis of the cervical lymph nodes. (After Eisendrath, *Surgical Diagnosis*, p. 171, by permission of W. B. Saunders Company.)

M, Uppermost node along internal jugular vein. *T*, Tonsillar gland. *S*, Submaxillary nodes. *V*, Internal jugular vein. *C-P*, and other lymph nodes in cervical region.

tioned earlier in this chapter. Infections of the tonsil drain into the tonsillar gland; infections of the floor of the mouth drain into the submental glands; from the adenoids into both the tonsillar gland and the internal jugular group, the latter also receiving lymphatic drainage from the ear. These various lymph glands communicate with others in the cervical region and in some cases

with the supraclavicular group. It is commonly taught that lymphatic drainage in the lung is from the periphery to the hilus, although Neff (1933) has described a number of cases of central pneumonia in children where extension from the hilus toward the periphery occurred. Winternitz (1920) definitely established that

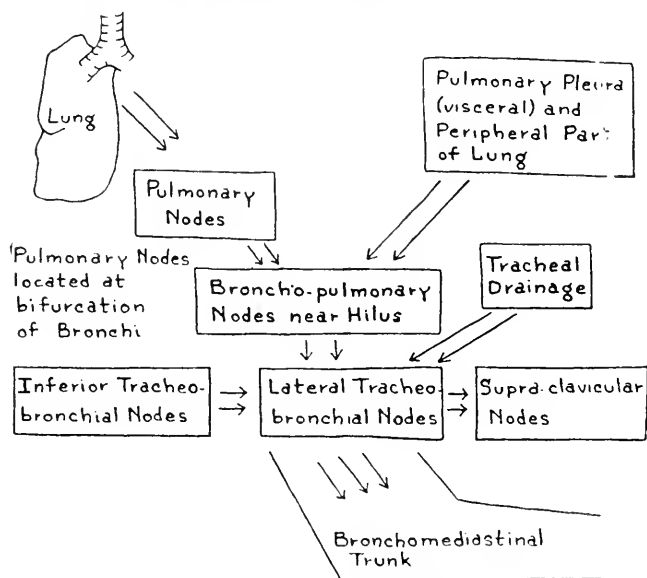


Fig. 3.—Pulmonary pleural, and tracheal lymphatic drainage.

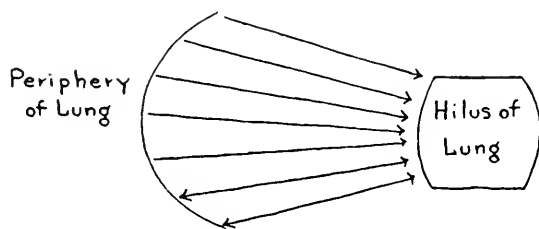


Fig. 4.—Drainage of periphery of lung.

infection of the upper respiratory tract may extend down the lymphatics of the tracheal wall and lead to acute pulmonary inflammation called pneumonia.

HEMATOGENOUS EXTENSION.—An excellent example of hematogenous spread of infection is seen in miliary tuberculosis. Here some focus of infection ruptures into the blood stream and the

organisms are showered throughout various tissues. In endocarditis and also in cases of thrombosis associated with infection, mycotic emboli are occasionally broken off and carried by the blood stream to other parts of the body. The new foci of infection are called metastatic foci.

DIRECT EXTENSION.—This method of spread of infection is seen in gas gangrene where the process extends from one muscle cell to another. While in abscess formation the initial infection is frequently of metastatic origin yet the enlargement of the abscess is due largely to direct extension.

Because fascia and tendon sheaths act as barriers to direct extension through them, abscesses take the course of least resistance and spread along fascial planes and tendon sheaths. Direct extension of a retropharyngeal abscess along the prevertebral fascia and of the spread of infection by direct extension in the submaxillary subcutaneous tissue is illustrated in Fig. 1.

Factors Involved in Pulmonary Infection.—It is obvious that infection of the lungs may result from aspiration of contaminated food material such as raw milk down the trachea, by extension along the surface or within the walls of the trachea, through the lymphatics from the cervical region, through the blood stream, from contact of the pleura with an infected pericardium or diaphragm, by direct trauma such as occurs in chest injuries or by direct extension of infection from the chest wall as might occur in an infected malignancy of the breast that had eroded into the pleural cavity. Small abscesses of the lung, located at the surface, may rupture into the pleural cavity and give rise to *empyema* or pus in the pleural cavity. It should be remembered that destruction of surface barriers by malignant growths offers excellent portals of entry for infection.

LOBAR PNEUMONIA.—Hadfield and Garrod (1938) give an excellent summary of important research bearing upon lobar pneumonia. They state that Blake and Cecil (1920) definitely settled the route of infection as being via the respiratory tract and not by hematogenous or lymphogenous routes. Their results and those of Stillman (1930) make it seem improbable that a downward spread on the mucous membrane occurs but that instead the organisms may be carried in by small inhaled droplets which penetrate the lung deeply before coming to rest during inspiration. Hadfield and

Garrod call attention to the accumulation of evidence showing that individuals developing lobar pneumonia have a pre-existing humoral immunity and perhaps an allergy for the pneumococcus. This may seem paradoxical but would fit in with the allergic hypothesis.

The conclusion of Blake and Cecil that the primary focus occurs at the hilus of the lung and that the infection spreads from the primary focus outward along the perivascular and peribronchial lymphatics has been challenged by Loescheke (1931), Terrell, Robertson, and Coggeshall (1933) and Gunn and Nungester (1936). The newer work indicates that the primary focus is usually near the periphery of the lung and that the infection spreads from the periphery toward the hilus in a wave of edema fluid that is swarming with pneumococci and later invaded by leucocytes. Apparently in the dermal pneumonia, as described by Goodner, edema fluid gravitates down the flank of the rabbit.

In producing experimental pneumonia, Gunn and Nungester suspended the pneumococci in mucin for intrabronchial injection. The mucin provides a nidus in which the pneumococci can begin to multiply. The mucin holds them together and protects them from phagocytes. These experimental results fit in with one concept of lobar pneumonia which postulates the existence of a nidus in which the pneumococci can begin to multiply. Lindau suggests that such a nidus consists of accumulated secretion which may be caused by chilling of the body or other factors. It has been shown experimentally that the application of cold to the skin causes hyperemia of the respiratory tract and also increased mucous secretion. Since pneumonia occurs quite frequently after operation on the upper abdomen, it is suggested by Coryllos and Birnbaum (1928) that a plug of mucous secretion in a bronchus is the nidus that aids the bacteria to initiate the pneumonia.

Factors Involved in Intestinal Infection.—Infections in the nose and throat may extend not only to the lungs, but also the swallowing of pus, whether it be coughed up from the lungs or come as a discharge from a sinus, etc., may lead to infections of the intestinal tract. This occurs quite frequently in tuberculosis where tuberculous sputum is swallowed.

As material passes from the region of the tonsillar ring along the intestinal tract, it is interesting to note that it encounters an-

other ring of lymphoid tissue near the ileocecal valve. Lymphoid tissue is scattered along the mucous membrane of the entire intestinal tract, but opposite the attachment of the mesentery and at the lower end of the ileum are rather extensive areas of it called "Peyer's patches." Peyer's patches are commonly the site of extensive ulceration in typhoid fever and in intestinal tuberculosis as well as in ileocolitis. Below the ileocecal valve is a lymphoid vestigial organ, the vermiform appendix.

ULCERATION OF INTESTINE.—In some cases of *tularæmia*, *agranulocytic angina*, and in *meliodosis*, there occurs extensive ulceration of the entire alimentary canal. Davis (1928) has reviewed his own work and that of others on the lymphatics of the respiratory and intestinal tract. He observed extensive infiltration of plasma cells in the upper respiratory mucous membrane under the epithelium of the crypts and along strands of connective tissue. Infiltrations of similar cells were observed by Aschoff in his studies of the appendix. Davis seems to feel that these plasma cells are indicative of either chronic or at least frequent inflammation. Goodpasture's (1937) work suggests that the *young* plasma cell may function as a host cell for certain bacteria.

The walls of the intestine may become infected directly through the mucous membrane by specific pathogenic organisms such as *E. typhosa* or the tubercle bacillus, or the infection may be due to the pyogenic organisms present, and the portal of entry may be through erosions from various causes as suggested by Ivy (1920, 1925). The infections may be hematogenous or lymphogenous in origin. Malignancy also is responsible for secondary infections. Direct extension from an abscess existing in some organ or mass to which the intestine has become attached by inflammatory adhesions may lead to invasion from the peritoneal side.

ULCER, PANCREATITIS, CHOLECYSTITIS.—The anatomical relationships near the pylorus of the stomach are quite important since *ulcer* commonly involves the cap or the walls of the duodenum just below the cap. The presence of ulcer interferes with the normal functioning of the gastrointestinal tract in various ways, is a source of mild or severe hemorrhage and is a defect in the intestinal wall that may lead to perforation and peritonitis. It should be remembered that the secretions from the liver and pancreas enter the duodenum as a rule through the ampulla of Vater. A stone lodging in

the latter may traumatize the tissue and infection result. Opie, many years ago, showed that acute *pancreatitis* (inflammation of the pancreas) may result from mechanical blocking, infection and the forcing of bile up into the pancreas. Inflammation of the gall bladder is called *cholecystitis*. This is usually due to extension of infection from some focus in the body and may in some instances result in multiple abscesses of the liver. A knowledge of the regional lymphatics and of the anastomoses of the portal circulation is of considerable value in visualizing the possible complications of infections of tissue drained by them.

APPENDICITIS.—Inflammation of the appendix is of rather frequent occurrence. While theoretically the route of infection may be hematogenous or lymphogenous, an examination of sections from inflamed appendices suggests that the infection frequently starts from injury to the mucosa. Fecaliths and small foreign bodies are found quite frequently and there are numerous reports of finding animal parasites within the lumen. Since the appendix is a blind vestigial organ, stasis of fecal material within it must be quite common. Thus one can see there is abundant opportunity for both mechanical and chemical injury to the mucosa while the feces in the region of the caecum is notoriously rich in bacteria. There is also the added possibility that vasomotor disturbances might cause vascular changes in the wall of the appendix and to some extent lower the resistance of the tissue.

The complication most feared by the physician is the rupture of an infected appendix. This permits pus from the appendix as well as fecal material with its rich bacterial flora to enter the peritoneal cavity and cause peritonitis. Measures usually employed to avoid such a catastrophe are surgical interference or bed rest with symptomatic treatment designed to keep the intestine at rest and to get the patient ready for operation if such is decided upon. By the term "get the patient ready for operation" is meant to employ measures that will tend to restore his physiological mechanisms to as near normal as possible. If he is dehydrated, he will be given fluids; if anemic, he might be transfused, etc.

IMPORTANCE OF BLOOD SUPPLY AT LOWER ORIFICES.—At the lower end of the intestinal tract and also in the genitourinary tract there is a rich blood supply that is an important factor in defense against infection since trauma is not uncommon, and infectious

agents are numerous. Abscesses in the region of the rectum do occur and may be exceedingly dangerous.

Factors in Genitourinary Tract Infections.—The female genitourinary tract is not uncommonly the seat of infections. These may be exogenous (from without), hematogenous, lymphogenous, or by direct extension. The cervix of the uterus and the vaginal walls are in contact with an extensive bacterial flora. The reaction of the secretions from the cervix and vagina may be irritating and the opportunity for fecal contamination is ever present. During childbirth the patient becomes exceedingly fatigued, there is always blood loss, the resistance of the tissues of the uterine walls and cervix is markedly lowered and the latter frequently lacerated. Thus there are many predisposing factors for infection in such conditions. Infection of the uterus may originate, and frequently does, from some focus within the body carried to the uterus by the blood stream or lymphatics.

Septic infection that complicates or follows delivery is called *puerperal sepsis*. Oliver Wendell Holmes called attention to its spread by the hands of the physician who goes from a case of septic infection to care for a woman in labor. Not infrequently one observes uterine infection in women who have attempted abortion. Such infections lead to pelvic peritonitis, general peritonitis or septicemia.

CYSTITIS, or inflammation of the bladder, is said to be more common in girl babies than in males of the same age. Since the colon bacillus is found frequently associated with this condition and since the female urethra is shorter than that of the male and commonly contaminated with fecal material in the diapers, it has been suggested that these factors account for the more frequent occurrence of cystitis in the female babies. The development of cystitis in older age groups is commonly associated with conditions that prevent the normal, free emptying of the bladder. This may be due to a cystocele in the female or to a hypertrophied prostate in the male or to tumor masses in either.

Cabot says that the so-called "*catheter cystitis*" is always caused by urinary retention with resulting congestion and edema of the bladder mucous membrane and the preparation of a "*soil*" for bacterial growth and development. In his opinion, proper catheterization prevents infection.

PYELONEPHRITIS.—In a like manner anything which prevents the normal function of the ureters as conductors of urine from the kidney to the bladder will result sooner or later in damming back the urine into the pelvis of the kidney and the development of a *pyelonephritis*. This may be unilateral or bilateral. When the latter occurs, uremic complications are possible. Cord changes such as are seen in multiple sclerosis and certain types of syphilis can cause paralysis of both ureters.

STONE IN THE URETER OR KINKS due to any one of many causes are commonly responsible for ureteral insufficiency and the preparation of a proper “soil” for the growth of infectious agents. These latter may come from the blood stream through the kidney or by some other route.

ACUTE NEPHRITIS.—Acute infections of the upper respiratory tract are sometimes followed by inflammation of the glomeruli of the kidney (acute glomerular nephritis) as well as infections of the heart or other tissues of the body. It is obviously quite serious for the genitourinary system, heart, central nervous system, or lungs to be impaired by acute infections. In fact, there are many delicate mechanisms that are of great importance in enabling the organism, as a whole, to maintain life in the presence of severe infections.

IMPORTANCE OF ELIMINATION.—Clinical experience has shown the great importance and imperative necessity of maintaining elimination by kidney, bowel, skin, and lung. It has also shown that confined pus disturbs the physiological balances in the body and that drainage or removal of such products of suppuration is necessary for recovery.

Mechanical Factors of Safety.—Meltzer (1906-07) called attention to many mechanical factors of safety in the animal organism. A large amount of lung, liver, and kidney may be rendered functionless or destroyed by infection or the spleen may be entirely removed without fatal termination. Undoubtedly there are many more islands of Langerhans present in the pancreas than are needed for adequate insulin production. There is also more thyroid, ovarian, testicular, or adrenal tissue than is necessary for minimum requirements of the individual.

Physiological Mechanisms.—Some of the physiological mechanisms that are important in resistance to infection are those

regulating the body temperature, production and distribution of blood, and the mechanisms regulating the acid-base and water balance.

Locke (1937, 1939) has worked out indices of fitness to resist infection of the respiratory tract based upon the relative efficiency of the temperature control mechanism in rabbits and of the capacity of oxygen replacement during exercise in man. He defines fitness as ability to support a forced performance of work at an effective rate of speed. In his opinion lack of fitness is a predisposing factor in such infections as pneumonia and the common cold.

It is quite evident that both the central and vegetative nervous systems are of considerable importance in immunological phenomena since functional change is dependent upon nervous as well as hormonal and direct stimulation. Pain of itself may lower resistance through resulting fatigue of the patient or on the other hand may protect a patient through forcing the immobilization of part or all of the body. Recent investigations involving the use of diathermy and also of the malarial parasite in the treatment of paresis suggest that fever favors phagocytosis of spirochetes by cells of the reticulo-endothelial system.

The use of hot wet dressings is still one of the important measures employed in the treatment of acute septic infections. When such an infection occurs upon an extremity, the physician shows his interest in the local blood supply and buffer mechanism of the blood by elevating the extremity, thus permitting of removal of venous blood by gravity and at the same time favoring an increased arterial supply. The latter supplies oxygen, leucocytes, antibodies, and an excellent group of buffer substances consisting of hemoglobin, the serum proteins, and alkalies. The physiologist considers hemoglobin the most important buffer of all.

When the physician examines a patient suffering from severe septic infection, he makes a note of whether the skin shows normal turgor or whether there is evidence of dehydration, and he frequently determines the CO_2 combining power of the patient's blood to ascertain the condition of the alkali reserve. Both dehydration and lessened alkali reserve favor infection and are combated by the administration of fluids and alkalies or some substitute that helps restore the normal alkali reserve. The state of lowered alkali reserve is spoken of as *acidosis* while the presence

of an excess of alkalis is called *alkalosis*. Neither is desirable and hence both are to be avoided.

Finally it should be recalled that the liver, in addition to other things, is quite potent as a detoxifier of proteins; the spleen and lymph glands are efficient filters for pathogenic bacteria because they form part of the reticulo-endothelial system. The bone marrow supplies red cells and white cells, the former endowed with a potent oxygen carrier that is also a good buffer and the white cells are phagocytic in nature. The vascular system and the vasomotor mechanisms aid in maintaining a normal distribution of blood, and these and many other factors help maintain the normal integrity of every tissue cell in the body.

PRENATAL VS. POSTNATAL IMMUNITY.—The importance of normal physiological factors in resistance is emphasized by the discovery of Woolpert and his associates (1938, 1940, 1941) that fetal susceptibility of guinea pigs to influenza virus is changed at birth to resistance. This suggests that in some unknown way the immunity is associated with postnatal circulatory and respiratory readjustments.

IMPORTANCE OF CONSIDERING THE BODY AS A WHOLE.—Since the cells of the body are protoplasmic in nature and since protoplasm is an intricate mass of dynamic colloidal systems, it is very essential to consider the "body as a whole" in attempting to understand infection and resistance. It would seem obvious that while circulating antibodies are of value in the body's defense against infection, except for antitoxins their role and relative importance have been considerably exaggerated. In diagnosis, however, they have won for themselves a place of great importance.

UNDERLYING PRINCIPLE OF THERAPY.—When a patient with a beginning pyogenic infection is treated by a physician, the idea behind the therapy is to maintain the local tissue mechanisms of defense at an optimum and thus encourage localization or prevent extension by any of the possible routes of dissemination. In the case of pelvic peritonitis the head of the bed may be elevated, thus enlisting the forces of gravity while other measures are being instituted. Generalized peritonitis is exceedingly dangerous owing to the large area of peritoneal surface through which absorption of toxic substances and infectious agents occurs and also because of the danger of bowel paralysis and complete suppression of urine

that may result. The physician favors the body's defense by measures designed to prevent the body temperature from going either too high or too low, establishing drainage, encouraging elimination of toxic substances through natural channels, maintaining an adequate water balance, blood volume and alkali reserve, since these are essential physiological mechanisms of defense. A quiet environment is also necessary since psychic stimulation may lead to expenditures of energy that is needed for recovery.

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CHAPTER V

THE RETICULO-ENDOTHELIAL SYSTEM

It is interesting to note that the concepts we now hold as to cellular immunity have their roots buried deeply in experimental biology, chemistry, and pathology. It was the zoologist, Metchnikoff, who about 1883-84 extended the then existing knowledge of the distribution and functions of amoeboid cells throughout the body and classified them into two groups which he named *macrophages* and *microphages*, respectively. He grouped together the fixed phagocytes which he found in the liver, spleen, lymph nodes, and in the central nervous system and the large free mononuclear phagocytic cells of the circulating blood and named them *macrophages*, while the neutrophilic leucocytes of the blood he called *microphages*.

It was his opinion that the macrophages were concerned with the phagocytosis and digestion of dead and foreign animal cells and debris while the function of the microphages was to engulf and destroy bacteria. The cellular enzymes which carried out the digestion of the phagocytized material he named *macrocytase* and *microcytase*, respectively, to indicate the type of phagocyte which produced them. These enzymes have been studied rather extensively by Opie (1906-1910). He found that the macrophages contain an enzyme that resembles pepsin in that it acts best in an acid medium while the microphages contain a trypsin-like enzyme that works best in an alkaline medium.

Before Metchnikoff began his work on phagocytosis, a great deal had been learned about the manufacture of dyes and their use in histology. According to Conn (1933) it is well established that Hill employed carmine in his histological studies of plant tissues as early as 1770 and there are claims made that either Sarrabat (1733) or Reichel (1758) were the first to use stains in histological work. About 1854 William Perkin prepared, for the first time, an anilin dye. The discovery of various other dyes now used in biology quickly followed.

According to Jaffé (1938), it was Ehrlich's (1879) studies on the chemical constitution and cellular affinity of dyes that were re-

sponsible for the great progress that has been made in our knowledge of the phagocytic cells of the body. Some of these important contributions may be listed as follows:

1. He was probably the first to recognize the distinction between acid and basic dyes.
2. He used them to differentiate between acidophilic and basophilic leucocytes.
3. He prepared what he called "neutral stains" with which he demonstrated the existence of neutrophiles.
4. He prepared benzidine dyes such as trypan blue and triphenyl methane dyes such as pyrrhol blue and isamine blue that were later used by Bouffard (1906) and Goldmann (1909) in their important works on vital staining and are now employed extensively in experimental hematology.
5. He prepared neutral red which has been used by Metchnikoff, Sabin and others in supravital studies of amoeboid cells.

By means of the acid colloidal dyes of Ehrlich, Goldmann (1909) demonstrated the significance of vital staining. In 1913 Aschoff employed lithium carmine, trypan blue, pyrrhol blue, and other dyes in a study of the extensive system of phagocytic cells scattered throughout the body. Purely upon the basis of the capacity of the various cells to take these acid colloidal dyes and store them, he divided the system, which he named *reticulo-endothelial*, into six groups. In the following arrangement in which the order given by Mann and Higgins is reversed, group one contains the most actively phagocytic cells while the cells of group six are almost devoid of phagocytic powers.

1. The monocytes of the blood (Plates I and II)
2. Wandering cells of the connective tissue called clasmatoocyte by Ranvier, polyblast by Maximow, and macrophage by Metchnikoff (Plate II)
3. The living cells of the blood sinuses of the spleen, the lymph sinus of the lymph glands, the blood sinuses of the liver, bone marrow, suprarenal cortex, and hypophyses (Plate III)
4. The reticular cells of the splenic pulp and cords of lymph nodes
5. Fibrocytes
6. The ordinary endothelial cells of the lymphatic and vascular system

Mann and Higgins suggest that the reactions to vital dyes of the fibrocytes and ordinary vascular and lymphatic endothelium are so slight that these two groups could well be eliminated. Jaffé reviews the various theories that have been offered to explain vital staining. It is evident from his discussion that there is great difference of opinion. A concept that is held by Hadfield and Garrod and many others is that vital staining consists of phagocytosis and concentration of the colloidal dye particles and that it differs from simple staining in this respect and also in that the process of osmosis is not involved as it is in simple staining. It should also be added that the living nucleus is not stained in vital staining.

According to Hadfield and Garrod (1938) the discovery that the cells of the reticulo-endothelial system come from undifferentiated mesenchyme and that there is actually present in the tissues of the adult, ancestral cells of the embryonic mesenchyme having totopotentialities should be credited to Maximow.

The *undifferentiated mesenchyme* in adult tissues is described as a cell syncytium in which it is difficult to make out cell boundaries. The cytoplasm of the syncytium is pale and slightly neutrophilic. The nuclei are pale, but there is usually no nucleolus. Hadfield and Garrod say that in the unstimulated organ the cell syncytia are inconspicuous and lie on the basement membrane of the lymph or blood sinuses. When the cells are stimulated, they swell and develop a cell outline. Such cells are called reticulum cells (Plate III, Fig. 4), and it can be established that they can be divided into two types only, the so-called littoral cell that does not contain argyrophil fibrils in the resting state while the other does. Both cells may free themselves from the sinus walls and become motile histiocytes (Plate III). These have been called polyblasts by Maximow, macrophages by Metchnikoff, and clasmatoocytes by Ranvier and others. Downey's *Handbook of Hematology** contains splendid chapters on the fibroblasts and macrophages and on the fixed system of histiocytes in the liver by Bloom (1938) and Mann and Higgins (1938), respectively, while Jaffé gives an excellent discussion of the reticulo-endothelial system. The supravital method of studying blood cells is well presented by Cunningham and Tompkins (1938).

It should be remembered that there are conflicting theories held regarding the origin of cells found in the peripheral circulation

*Paul B. Hoeber, Inc., New York, 1938.

PLATE III.—CELLS OF RETICULO-ENDOTHELIAL SYSTEM SHOWING PHAGOCYTOSIS
OF INDIA INK

1. Omentum of rat injected with India ink. H—Histiocyte.
2. Alveolar wall of lung of guinea pig after inhalation of coal dust. S—Septal cell.
3. Bone marrow of guinea pig after India ink injection. R—Reticular cells.
4. Reticulum cells of the spleen of a dog (not injected with India ink).
5. Sinusoid of the liver of a dog after India ink injection showing, K—Kupffer cell containing India ink; E—Endothelium of sinusoid; L—Lymphocyte; S—Sinusoid; Er—Erythrocyte.

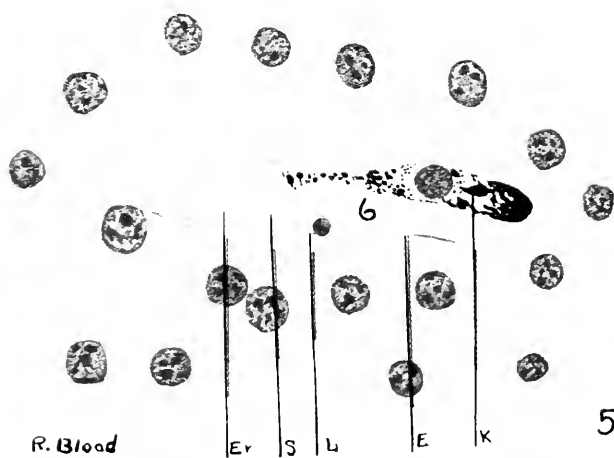
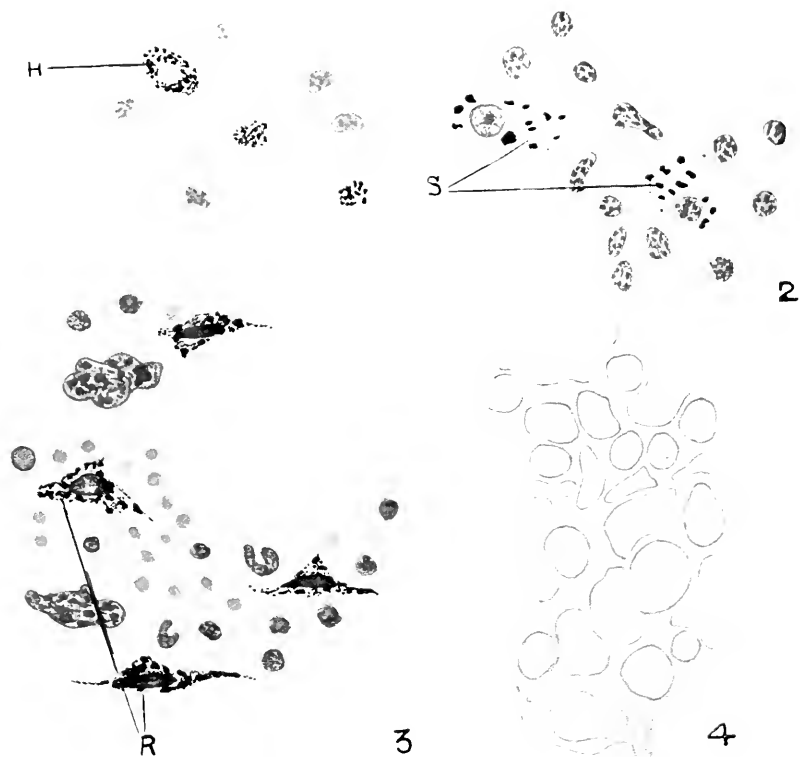


PLATE III.

(See explanation on opposite page.)

in health and disease as well as the phagocytic cells of the tissues and the various types of cells observed in inflammatory exudates. The *monophyletic theory* assumes that the embryonic mesenchyme gives rise to a stem cell, called by Pappenheim the lymphoidocyte, which is endowed with in toto-potentialities. The environment in which it finds itself determines the type of cell which develops from it. In contrast to this is the *polyphyletic theory* which assumes that the embryonic mesenchyme gives rise to stem cells endowed with different potentialities: one is destined to give rise to granulocytes, another to lymphocytes, etc., regardless of the environment.

The adherents of both theories agree that the stem cells originate from cells that are of mesenchymal origin and that the adult mononuclear phagocytic cell of perhaps major importance in the body's defense has a peculiar affinity for trypan blue. The majority of workers are willing to call this cell a clasmatoocyte or histioocyte. Sabin et al. formerly (1925) divided the large mononuclear phagocytes into two groups. One group which has an affinity for trypan blue she called clasmatoocytes. These, she thought, originate intravascularly in the bone marrow, liver, spleen, and lymph glands and migrate into the tissues, to become the tissue phagocytes. The second group, which has little affinity for trypan blue, she called the monocyte. These, she thought, originate extravascularly from reticular cells in the same tissues. They enter the blood stream and constitute the monocytes of the peripheral circulation although they too are found in the tissues. More recently Sabin (1932) has come to regard the monocyte and clasmatoocyte as one and the same cell exhibiting difference in appearance as a result of the kind of material it has ingested. The various types of cells found in the blood and tissues are illustrated in Plates I, II, and III.

These show the monocytes and clasmatoocytes of Sabin as they appear in supravital preparations where neutral red is used to demonstrate granules and vacuoles and Janus green to stain any mitochondria present. It will be observed that the monocyte has a kidney-shaped nucleus with a rosette of neutral red granules in the cytoplasm within the *hof* of the nucleus. These granules surround the centrosphere. The vacuoles containing neutral red are more or less peripherally arranged. The clasmatoocyte has an oval,

The functions of the cells of the reticulo-endothelial system may be summarized as follows:—

1. The macrophages engulf senile and injured red cells and erythrocyte fragments and thus participate in their removal from the circulation, from areas of infection, and from the tissues where extravasation has occurred.

2. They phagocytize and destroy dead and dying leucocytes and injured tissue cells.

3. They phagocytize inert foreign material such as deeply inhaled dust and also carbon particles, India ink, and acid colloidal dyes gaining entrance to the blood stream, thus removing them from the circulation.

4. In inflammatory and degenerative conditions of the nervous system phagocytic cells, according to Hadfield and Garrod, remove the free myelin and its disintegration products.

5. According to Bunting (1938), in certain chronic infections the macrophages form the epithelioid cell which in addition to its property of phagocytosis is able to secrete a precollagenous reticulum (Miller) that aids in localizing infection.

6. The macrophages may fuse and form the foreign body giant cell and thus be able to interpose a mass of protoplasm between the tissue cells and a body too large to be engulfed.

7. According to Mann and Higgins (1938) and Hadfield and Garrod (1938) one of the functions of the fixed histiocytes in the liver is the metabolism of the blood pigments leading to the retention and return to the bone marrow of the iron liberated from the disintegrating red cells and the excretion of the iron-free portion as bile pigment.

8. These same authors also state that the liver histiocytes together with the liver cell seem to act as a functional unit in lipid metabolism.

9. According to Sabin (1939) and Bunting (1938), there is some evidence suggesting that the cells of the reticulo-endothelial system help in maintaining the normal composition of the plasma proteins.

10. There is a great deal of evidence indicating that antibodies are produced perhaps entirely by cells of the reticulo-endothelial system. This view was held by Metchnikoff.

When one injects a cellular antigen into the animal body, the antigen is rapidly removed from the point of injection and appears in the blood stream. From the latter it is removed by phagocytic cells of the reticulo-endothelial system. If one uses an antigen such as avian red cells which can be readily identified microscopically, one can trace these to the Kupffer cells of the liver and fixed tissue cells of the spleen, lymph glands, etc., by studying properly prepared sections of these tissues. After an interval of several days, specific antibodies for avian red cells, or the particular antigen employed, appear in the blood.

Many attempts have been made to determine experimentally where the antibodies are produced. One of the most successful methods of investigation has involved an attempted blockade of the cells of the reticulo-endothelial system by injecting inert particulate matter such as India ink. An excellent review of the subject is given by Howell (1928). She concludes that while the evidence points to the reticulo-endothelial system as the source of antibodies, the question is not definitely settled.

Since the publication of Howell's paper Cannon, Baer, Sullivan, Webster (1929), Kroo and Janesó (1931) have published results that quite definitely implicate the reticulo-endothelial system as an important source of hemolysin and perhaps other antibodies. Cannon et al. give a critical review of previous work and point out many sources of error. They find that the manner of administration and the amount of antigen injected affects the liberation of hemolysin following blockade. They also determined that an incomplete blockade increases the amount of hemolysin production while a complete blockade inhibits antibody production. Their work is carefully controlled and apparently supports the theory that antibodies are produced by the reticulo-endothelial system. Plate III shows fixed phagocytic cells in various tissues. They have taken up India ink injected intravenously to produce a blockade of the reticulo-endothelial system.

11. The cells of the reticulo-endothelial system form the main defense mechanism of the body against infectious agents after the latter have once gained entrance. This is discussed more extensively in Chapter VII. Bunting (1938) says that there is suggestive evidence that they may aid in preventing invasion of the body.

Phagocytosis.—Both macrophages and microphages have to be considered in any discussion of phagocytosis. From the standpoint of the defense of the body against disease the engulfment of bacteria or other infectious agents by amoeboid cells raises the following three important questions: (1) How do the phagocytes and the bacteria get together? (2) What is the mechanism of engulfment? (3) What disposition is made of the engulfed organisms?

It would seem evident that the fixed histiocytes, as e.g., the Kupffer cells of the liver, are concerned with the removal of bacteria and other substances brought to them by the circulation while the free histiocytes and neutrophils can be mobilized wherever needed. The *mechanism of mobilization* has been investigated quite extensively. It is known that many substances, among them bacterial protein, attract phagocytic cells. This is called *positive chemotaxis*. When phagocytes are repelled it is called *negative chemotaxis*. Attention is called in Chapter III to Menkin's discovery of a leucocyte-attracting substance in inflammatory exudates. He has obtained this nitrogenous substance in crystalline form and named it "*leukotaxine*." It is thought that amoeboid cells move toward the source of a chemotactic substance because of the change that the latter substance effects in the surface tension of the phagocyte. Among other theories offered in explanation of the observed attractions of phagocytes are that osmotic forces govern their movements, that the difference in the surface potential between the amoeboid cell and the bacteria is the important factor in chemotaxis.

Menkin seems to have shown that the hydrogen ion concentration is an important factor in determining the prevailing type of cell in an area of inflammation although it is not the chemotactic factor. Apparently there is no theory that explains adequately the mechanism of attraction or tropism of free amoeboid phagocytic cells in the body. In an interesting report by Mallery and McCutcheon (1940) they say that the motility of leucocytes obtained from acutely ill individuals may not only be diminished but the leucocytes show a less direct approach to the bacteria.

In regard to the factors that play a rôle in the engulfment of bacteria by phagocytic cells Mudd (1927), as well as others, thinks that the presence of a film of denatured globulin on bacteria or

particulate matter makes it possible for leucocytes to engulf them more readily. As will be shown in another chapter there is a great deal of evidence indicating that when immune serum is mixed with homologous bacteria the latter specifically adsorb antibody protein (globulin) on their surfaces and that this film of globulin becomes insoluble in salt solution (denatured). This probably constitutes the process called *opsonification* or preparation of the cell for phagocytosis. Natural antibody acting in this manner is called *opsonin* while similarly acting antibody in an immune animal is called "bacteriotropin."

Some of the earliest investigations of the rôle that antibodies play in the process of phagocytosis were carried out by Denys and Leclef (1895), Mennes (1897), and Marchand (1898). They showed that immune serum increased phagocytosis and thought it was due probably to some action on the bacteria. According to Muir (1931) particulate matter such as charcoal, flour, powdered albumin, milk globules, and other materials, adsorb serum proteins and become nonspecifically opsonized.

In 1903, Wright and Douglas carried out an extensive investigation of the whole question and concluded that certain immune bodies which they called opsonins acted upon the bacteria and rendered them more susceptible to phagocytosis. In this work they used a modification of Leishmann's (1902) technique of studying phagocytosis. They concluded that normal opsonins are inactivated at 60° C. for fifteen minutes. In 1904, Neufeld and Rimpau discovered that immune opsonins are heat stable. They consider this an important difference between them and normal opsonins. Bullock and Western (1906) and Hektoen (1908) showed that normal opsonins could be specifically adsorbed and Chapin and Cowie (1907) showed that inactivated opsonin could be reactivated. This suggested that it was quite similar to Bordet's sensitizer or Ehrlich's amboceptor.

RÔLE OF COMPLEMENT.—Dean (1905, 1907) and others have shown that even the activity of immune opsonins (named bacteriotropins by Neufeld) is materially increased by normal serum containing complement. Sleeswijk (1908) concludes that both normal and immune opsonins have dual structures in that the activity of both is increased by complement. He suggests that

perhaps the reason complementary actions dominate the picture in the case of normal opsonins is the low concentration of the latter while the high concentration in immune sera reduces the activating action of complement to a mere enhancement of an effect which takes place in its absence.

Apparently surface forces play an important rôle in phagocytosis. For those interested in certain theories involving the free surface energy at the interfaces of bacteria and phagocytes, the papers of Fenn (1922, 1928) and Mudd, McCutcheon and Lueké (1934) are recommended as of interest.

In regard to the third question concerning the fate of ingested infectious agents a number of possibilities need be considered:

1. The cellular matter may be taken in and then ejected without apparent change.

2. After ingestion, living cells may be killed and digested, and any remaining residue ejected.

3. The ingested infectious agent may be transported within the phagocyte to some depot and destroyed immediately or after an indefinite period of time. This accounts for the presence of viable organisms in cultures from the spleen, lymph nodes, and other tissues when blood cultures are negative.

4. Goodpasture's work on the pathogenesis of typhoid fever suggests that young plasma cells may function as host cells for *E. typhosa*.

5. The phagocyte may be destroyed by the ingested organisms.

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CHAPTER VI

NATURAL AND ACQUIRED IMMUNITY

Natural Immunity.—SPECIES DIFFERENCES IN IMMUNITY.—The two infectious agents that cause cattle plague and chicken cholera respectively are unable, under natural conditions, to produce disease in man. Conversely, the infectious agents that cause syphilis, gonorrhea, Asiatic cholera, measles and a few other diseases peculiar to man, never, under natural conditions, produce these diseases in the lower animals. Dogs, cats and rats are relatively resistant to pneumococcus infection whereas man and guinea pigs are quite susceptible. Rats can ingest relatively large doses of botulinus toxin with apparent impunity whereas man and guinea pigs succumb following the eating of exceedingly small amounts. Coriell and Sherwood* found that cats possess almost solid immunity to strains of salmonella organisms highly virulent for mice as well as to strains of *B. anthracis*, *P. tularensis* and a strain of vaccine virus that is exceedingly virulent for rabbits. On the other hand they found cats moderately susceptible to trichina infections, and Leasure (1934) observed cases of fatal feline virus enteritis.

RACIAL DIFFERENCES IN IMMUNITY.—Occasionally susceptibility and resistance are found to correlate with racial differences. Algerian sheep are said to show a much higher resistance to anthrax than our domestic sheep and various races of mice differ in their resistance to a number of infectious agents. Bay-Schmith (1929) reports that diphtheria does not occur among the Eskimos although the Schick test indicates that the normal percentage of susceptibles exists among them. Sherwood, Nigg and Baumgartner (1926) observed a similar phenomenon as regards scarlet fever among full-blooded American Indians. They rarely have the disease although the Dick test indicates a high percentage of susceptibles in the age group of five to fifteen years. Toyoda, Moriwaki and Futagi (1930) have compared the percentage of positive reactors to scarlet fever toxin among comparable groups of adult Chinese and Japanese. The ratio of susceptibles within the two races is as 1 to 2, while the morbidity statistics show equal susceptibility to

*Unpublished work.

scarlet fever at birth and a ratio of 1 to 45 among adults. Scarlet fever is said to be unknown among the Eskimos, and it is interesting to note that Heinbecker and Irvine-Jones (1928) found no positive reactors to scarlatinae toxin among 53 Eskimos tested by them. These investigations *seem* to offer examples of racial immunity to diphtheria and scarlet fever respectively. There is a great deal of controversy over the interpretation of the results and more than one explanation of the phenomena.

Wells* (1933) made an extensive study of the bacterial flora of the throat of a fairly large series of Eskimos. He noted the presence of nonvirulent corynebacteria quite frequently in throat cultures. In one instance a transient virulence was observed. From his serological, epidemiological and bacteriological studies he feels warranted in concluding that the immunity to diphtheria observed among the Eskimos "is specific rather than the result of maturation phenomena." He admits, however, that his evidence is not conclusive.

ENDOCRINES AND IMMUNITY.—There is a growing interest in developmental physiology and anatomy. Research in these fields may ultimately result in a better understanding of the defensive mechanisms of the individual at different periods in his life cycle. Scammon (1925) and others have carried out extensive work in developmental anatomy. They have worked out interesting growth curves but have not studied the corresponding cellular and biochemical changes.

Recent work suggests that the suprarenal cortex regulates, by its secretions, the permeability of vessel walls. In other words this secretion determines what substances stay within the walls and what escape into the tissues. When one turns to the developmental curve for the suprarenals, it is interesting to note that Scammon (1925) and others have shown that the suprarenals increase rapidly during fetal life, weighing about 7 grams at birth. During the first two years of postnatal life they undergo a rapid diminution in size to about a third of the natal weight. This reduction in weight is due to an involution of the middle and inner cortical zones. From about the second year they gain very slowly in weight until middle or later childhood when accelerated growth is noted for a period and then a gradual growth until maturity.

*Wells, J. R.: Am. J. Hyg. 18: 629, 1933.

In view of the recent developments in the field of endocrinology and especially the work of Britton and Silvette, Swingle, Hartman and others on the function of the suprarenal cortex, one is encouraged to hope that correlative studies may result in an enhancement of our immunological and pathological concepts.

This possibility is suggested by the work of Lewis (1928), Jaffé and Marine (1924), Jaffé* (1926), Scott (1924) and Belding and Wyman (1926). Lewis reports that a dose of 200 M.L.D. of diphtheria toxin for a guinea pig has no effect upon normal rats but is lethal for suprarenalectomized rats. Jaffé calls attention to the conclusions of Stewart and Rogoff (1922), Rogoff and DeNecker (1926) and Rogoff and Ecker (1926) that suprarenalectomized rats do not show increased susceptibility to toxins if tested after having fully recovered. Jaffé's conclusions are summarized as follows: "(1) Recently suprarenalectomized rats, fully recovered from immediate operative effects are highly susceptible to small doses of typhoid vaccine; (2) that as late as five months after operation suprarenalectomized rats having *no gross suprarenal accessory tissue* are still susceptible to vaccine; (3) that suprarenalectomized animals *when compensated* as regards resistance, invariably possess gross cortical accessory tissue; (4) that in the absence of gross accessories, autoplasmic suprarenal transplants will protect suprarenalectomized rats against typhoid vaccine." He also states that "suprarenalectomized rats show a lowered resistance to natural infections."

Belding and Wyman (1926) confirmed and extended the work of Lewis. They report that suprarenalectomized rats are 2.5 times as susceptible to diphtheria toxin as normal rats. They conclude that suprarenal deficiency apparently renders less effective the normal mechanism of the rat for the elimination or destruction of diphtheria toxin.

Jungeblut, Meyer and Engle (1934) report that poliomyelitis virus is inactivated in vitro by biological products containing anterior pituitary-like principles and by cortical and medullary adrenal cortex hormone and that pregnancy urine preparation and adrenal cortex hormone exert the same effect on diphtheria toxin.

It would seem that these findings warrant further investigation since Molomut† (1939) cites considerable evidence indicating that

*Jaffé, H. L.: Am. J. Path. 2: 421, 1926.

†Molomut: J. Immunol. 37: 113, 1939.

removal of either the adrenals or the hypophysis does not affect antibody formation. He did find that anaphylactic shock could be produced in hypophysectomized cats but not in the normal controls.

AGE AND RESISTANCE.—It is generally recognized that in the clinical entity known as "status lymphaticus" where the thymus gland does not undergo its normal involutionary changes, the child is quite susceptible to intercurrent infections. Clinical experience has also established the fact that at both extremes of life the individual possesses relatively little capacity to combat the majority of infectious diseases. The relationship of age to immunological reactions is discussed at some length by Baumgartner (1934), but her paper refers only to the humoral elements (antibodies). In this connection the work of Sutliff and Finland (1932) is very suggestive. They investigated the bloods of individuals falling into various age groups relative to the content of agglutinating, mouse protecting and bactericidal antibodies for various strains of pneumococci. In regard to mouse protecting and pneumococidal antibodies Finland and Sutliff (1932) observed that they are relatively rare in the blood of children, relatively frequent in adults, and less frequent in elderly individuals. They regard the frequency curve for pneumococidal power as probably similar for all types, showing a peak in adult life.

That the process of aging is associated with increasing resistance to certain viruses is indicated by the work of Olitsky, Sabin and Cox (1936), King (1940) and Casals (1940). Of added interest are the reports of Culbertson and Kessler (1939), Morgan (1939), and Casals and Webster with rabies infection in mice. In later work Casals (1940) finds that in the case of rabies, the influence of age in immunizability is evident only under definite sets of conditions.

Warthin (1929) discusses the anatomical changes that take place from conception to old age and death, but does not attempt to correlate structural and functional change with the relative integrity of the defensive mechanisms of the body. In view of the apparent increase in interest in the relationship of the aging process to immunity these publications are timely and suggestive.

VITAMINS AND FOOD FACTORS IN RESISTANCE.—The importance of vitamin A in the defense of the body against infectious agents is

another subject over which there is a great deal of controversy. As regards the experimental animal there is abundant evidence indicating that this vitamin plays an important role in maintaining in a normal healthy state the epithelial coverings of the body. When animals are fed upon a diet deficient in vitamin A, there results marked pathological changes in the cutaneous system, epithelium of the eyeballs, paraocular glands and the respiratory epithelium. These changes, among them being extensive keratinization of the ocular and respiratory epithelium, are associated with a definite lowered resistance to invasion by pyogenic bacteria. After infection is established, the feeding of vitamin A does not result in a cure. Thus it would appear that its function is preventive in that it aids in maintaining a normal healthy epithelium which is resistant to invasion.

Whether vitamin A plays a similar role in man has been much debated. In an interesting discussion of this subject Eusterman and Wilbur (1932) present evidence bearing upon both sides of the controversy. They are quite conservative and state that further research is desired, yet they seem to think that it may play a prophylactic role in man similar to the one it plays in the experimental animal. For a more extensive discussion and a comprehensive bibliography of the subject the student is referred to an excellent symposium on vitamins (1932), to a discussion by Szent-Györgyi (1939), a report by the Council on Pharmacy and Chemistry and Council on Foods (1939) and to a paper by Mackie, Eddy and Mills (1940). While these papers present many new facts about vitamins, they throw very little light upon their role in immunity.

NUTRITION AND RESISTANCE.—Anderson and Fraser (1934) have studied the influence of nutrition on the natural immunity reactions of the blood and skin to bacterial toxins. They find that a calorie deficient diet decreases both the hemolytic activity of sheep serum toward rabbit erythrocytes and its complementing powers but it increases the agglutinating power of serum for *Br. abortus* (porcine). They find that intradermal toxin tests are not affected either by a deficiency diet or by a diet which includes cod-liver oil and calcium.

In regard to the effect of diet on inborn resistance in mice infected with *B. enteritidis* Webster and Hodes (1930, 1939) report that altering the diet did not affect the spread of infection in a

mouse population but their resistance measured in terms of the killing potency of the organism was altered. They conclude, however, that in exposed or infected populations of mice the mortality is conditioned, primarily, by the number of highly susceptible constituents.

These discussions of hereditary and nutritional factors are included in this chapter since they play a role in determining both the morbidity and mortality in large groups as well as in individual cases.

NEUFELD'S THEORY OF PHASES OF HEIGHTENED AND LESSENED RESISTANCE.—Neufeld and his colleagues, Etinger-Tuleczynska (1933) and Kuhn (1933), hold to somewhat different views. They explain the apparent immunity observed among survivors of experimentally infected mice as due to the testing of the animals during a highly resistant period. They seemed to think that there are periods of increased and decreased natural resistance. During an epidemic some individuals will be in the phase of heightened while others will be in a phase of lowered resistance. The former constitute the group of survivors whose immunity is ordinarily attributed to previous infection but which Neufeld regards as due to the fortuitous circumstance that they are in the phase of heightened natural resistance, whatever that may mean. Undoubtedly many factors operate. Hirschfeld (1927) thinks that he has demonstrated hereditary factors in natural immunity to diphtheria. His conclusions have been severely criticized by Snyder (1927), Rosling (1928) and others.

HEREDITARY FACTORS IN NATURAL IMMUNITY.—Webster and his colleagues, Burn and Pritchett, conclude from their studies of microbe virulence and host susceptibility that hereditary factors, microbe distribution, microbe virulence and dietary factors are all quite important in determining whether infection occurs, the severity, the development of the carrier state and also the duration and complications of the disease in mice and rabbits. Lambert (1932) reported that by selective breeding he could produce individuals more resistant to the organisms of fowl typhoid than those in the unselected stock.

Since the latter paper appeared, Irwin (1929, 1933) has published the results of four years' study of the role of inheritance

in resistance of various inbred litters of rats to *S. enteritidis* infection. Schott (1932) has also studied the effect of selection on the resistance of mice to *S. aertrycke* and Monreas (1932) of rabbits to *Br. abortus*, var. *suis*. These have all shown that under experimental conditions, inheritance is important both in resistance and susceptibility of mice and rabbits to infection. Similar conclusions have been drawn by Lewis* (1928) from an extensive study of experimental tuberculosis in guinea pigs. He says that his "observations agree very well with the older conception of an inherited predisposing constitutional diathesis as a significant factor in the incidence of tuberculosis."

These views are somewhat at variance with those of Topley, Wilson, Lewis and Greenwood (Topley, and Wilson, 1929, 1936) who conclude from a study of statistical data on host survival during epidemics that active immunity due to either recognized or subclinical infection is a more important factor in survival than selection of innately resistant animals. Similar conclusions have been drawn from Armstrong's studies on mice surviving nasal instillation of the St. Louis encephalitis virus. He found them relatively resistant to a second instillation and concluded they had become immunized.

Webster and Hodes (1940) point out certain sources of error in the experiments cited above and present very convincing experimental evidence in support of their previous conclusions that inheritance factors play an important role in survival. Their results cast grave doubts upon the conclusions of Topley et al. as well as those of Armstrong. They found that survivors were almost exclusively individuals known to be innately resistant and that there was no tendency for known susceptibles to become immunized through exposure before or during epidemic times. They grant the possibility that future research may show that survivors of an epidemic may develop through infections, what Webster and Hodes call a *luxury immunity*. They were unable to immunize susceptibles by giving them sublethal doses of mouse typhoid bacilli or St. Louis encephalitis virus by a natural route.

These observations may not invalidate the contention of Topley and Wilson that under field conditions the possession of an active

*Lewis, Paul A., Loomis, D.: J. Exper. Med. 47: 1437, 1928.

immunity in man due to previous specific infection influences the survival of many individuals during an epidemic.

ANTIBODIES AND NATURAL RESISTANCE.—Antibodies are specific biochemical substances which may or may not be present in the blood. They can be detected by the effect they have upon the specific chemical substances (antigens) for which they have an affinity. The antibodies, called antitoxins, neutralize their specific toxins and under proper conditions the toxin-antitoxin mixture may form a precipitate; the union of specific hemagglutinins to homologous red cells in the presence of electrolytes results in the agglutination or clumping of the cells. Likewise the union of antibody with homologous bacteria in the presence of electrolytes results in the agglutination of the bacteria.

When red cells or bacteria are united with their specific antibody, they become susceptible to dissolution or lysis by a normal enzyme-like constituent of blood called complement. A more extensive discussion of antibodies is given in subsequent chapters. Because of the specific relationship that exists between antibody and its bacterial antigen, antibodies are regarded as specific immunity factors. Webster and Hodes (1939) conclude from their studies of mouse typhoid that specific immunity factors play a *major role* in infections with a high morbidity and a low mortality rate and a *minor role* where the infection gains access to a herd and spreads for the first time or when the infection is associated with a high mortality rate.

Irwin and Hughes (1933) in their study of inheritance immunity in rats noted that death of infected animals seemed to be correlated with absence of germicidal power in their blood. While this suggests a possible correlation between immunity and antibody, yet many exceptions have been reported in the literature. There is some evidence that the passive transfer of antibodies from mother to offspring through *mammary secretions* or through the *placenta* may confer temporary protection to the latter. This is supported by the following research: Theobald Smith (1907) demonstrated that in guinea pigs transient immunity can be transmitted from mother to offspring through the milk. He has also shown that the newborn calf acquires antibodies through the colostrum from the mother.

Kuttner and Ratner (1923) confirmed the observation of other investigators who found that diphtheria antitoxin passes through the human placenta from mother to child but that it is not present in the colostrum or breast milk of the mother. Kuttner and Ratner call attention to the anatomical variations of the placenta in different animals. There are *four main types of placentas*: those with one, two, three, or four cell partitions or layers between the fetal and maternal blood. In man and rodents there is only one connective tissue layer while ruminants have three and other animals such as swine have four layers.

Acquired Immunity.—It is now generally recognized that individuals recovering from certain acute infectious diseases such as typhoid fever, scarlet fever, measles, mumps, poliomyelitis and smallpox develop a lasting specific immunity as a result of the infection. To a certain extent this is true also of tuberculosis. In the case of syphilis the patient is immune to reinfection with *Treponema pallidum* so long as he has syphilis, but loses his immunity after he has been cured of the disease. Individuals who recover from influenza or pneumonia or from various pyogenic infections owe their recovery to the operation of immunity mechanisms but their increased resistance is of short duration.

The exact mechanism of acquired immunity is not clearly understood. While antibodies are of some importance in resistance, it has been established that a correlation between antibody titer and resistance does not always exist. Hadfield and Garrod (1939) state that the development of lobar pneumonia seems to require a pre-existing humoral immunity; Coriell and Sherwood (1940) found no virucidal antibodies in cats that were immune to vaccine virus highly virulent for rabbits; Kessel and Stimpeet (1941) studied poliomyelitis in a large series of monkeys and compared immunity with antibody titer. They found a very definite lack of correlation since it was present in only 50 per cent of their convalescent monkeys. Further consideration of immunity mechanisms will be presented in later chapters.

IMMUNIZATION AGAINST VIRUSES AND RICKETTSIAE.—The phenomenon of acquired resistance or active immunity to infection was perhaps first observed by the Chinese. They practiced nasal inoculation of smallpox virus and the production of the disease

in individuals in order to render them immune. A procedure of protective inoculation (into skin) was introduced into England in 1717 by Lady Montagu who had learned of it while in Constantinople.

In 1798, Jenner published his brilliant discovery that inoculation of the human being with *vaccinia* or cowpox virus leads to the development of a lasting active immunity to the virus of smallpox. Thus was introduced the term *vaccination* as well as the principle of immunization with an infectious agent of low virulence.

While at first, vaccination against smallpox encountered great opposition and was fraught with the danger of secondary infection, it has, nevertheless, achieved brilliant results. This is indicated by Turgensen's statistics for Sweden, cited by Zinsser and Bayne-Jones (1939). He states that during the 25 years preceding the introduction of smallpox vaccination (1776-1801) the death rate due to this disease was 2,050 per million inhabitants. During the transitional period (1801-1810) it was 680, and after vaccination became compulsory in 1810, it dropped to 169 per million inhabitants. In the Kansas City epidemic of 1921, there were 1,090 cases with 222 deaths. The deaths occurred among non-vaccinated individuals only.

Great progress has been made in the preparation of a satisfactory vaccine. Calves have been used almost exclusively in its preparation although Rivers (1933-1939) states that it is now possible to prepare a vaccine of more assured bacterial sterility by cultural methods. Gallagher and Wolpert (1940) describe a method of vaccine preparation in the rabbit fetus. A comprehensive discussion of methods of vaccine preparation and immunization against smallpox, rabies, and rickettsiae is given by Zinsser and Bayne-Jones (1939) and by Zinsser, Robinson, and other contributors to the Harvard symposium on virus and rickettsial diseases (1940). While it is generally recognized that living, attenuated virus vaccines are the most potent ones, yet there are many, according to Zinsser (1940), who feel that killed virus and killed rickettsiae have some immunizing value providing sufficiently large doses are employed. Veintemillas (1939) reports that the formalin-killed suspension of Mexican rickettsiae prepared in accordance with the methods of Zinsser and Castaneda (1931) has been found of prac-

tical value for large scale immunization against typhus fever. The subject of immunization against the virus of poliomyelitis is discussed in a later chapter.

Burnett, Andrews and others have shown that immunity can be induced in animals and man by the inoculation of influenza virus by various means. There is some evidence (Burnett, 1937, 1938) that cultivation of influenza virus on chick embryos alters its virulence without impairing its immunizing properties. Recently Horsfall (1940-41) has prepared a complex virus vaccine using influenza A virus and canine distemper virus X. The antibody response in ferrets and man has been much more adequate than that from any of the other preparations previously used. If this vaccine proves to be as successful in man it will be possible to immunize both military and civil populations against this very serious disease. Since there are a number of strains of influenza virus (Smith and Andrews, 1938) it would seem that for general use a polyvalent vaccine or one with broad antigenic powers should be required.

Jenner and his colleagues apparently failed to recognize the underlying principles in the use of attenuated smallpox virus as an immunizing agent. These principles and their broad biological significance were disclosed by Pasteur in his studies made a century after Jenner in 1879-80.

IMMUNIZATION WITH ATTENUATED BACTERIA.—By accident or design Pasteur discovered that chickens inoculated with old or attenuated cultures of the organisms of chicken cholera became immune to subsequent infection with virulent strains. He had previously noticed that animals recovering from experimental anthrax were immune. It is quite likely that Jenner's discovery was a factor in enabling Pasteur to grasp the broad fundamental principles involved and to establish the science of preventive inoculation or immunization upon a firm foundation. His successful and spectacular immunization against anthrax and rabies, using attenuated bacteria and virus respectively, placed protective vaccination upon a firm foundation.

Since Pasteur's time the French school has clung tenaciously to the principle of immunization with living attenuated organisms as evidenced by the present use of the B.C.G. (*Bacillus* of Calmette

and Guérin) vaccine in their campaign against tuberculosis. Attention is called in Chapter XXVII to the results of studies by Park and his colleagues on immunization against tuberculosis with B.C.G. vaccine. They seemed to find the vaccine of little value in human immunization.

It is possible that a new acid-fast organism causing tuberculosis in wild voles (small rodents) in England may be superior to B.C.G. The organism originally isolated by Wells (1937) has been cultured on Dorset's egg medium and employed by Wells and Brooks (1940) to immunize guinea pigs against virulent human tubercle bacilli. In their experiments they employed vaccines made from 2 strains of vole acid-fast bacteria, to immunize two groups of guinea pigs: a third group was immunized with B.C.G. vaccine while Group 4 consisted of unvaccinated controls. Five months after vaccination, Group 1 was injected with virulent human tubercle bacilli and Group 2 with virulent bovine tubercle bacilli. Group 3 was divided into 2 lots, one receiving virulent human and the second virulent bovine tubercle bacilli. Similar treatment was given to Group 4. The experiments were terminated after 11 weeks because of the war. The results indicate that the vole vaccine gave *almost* complete protection for Groups 1 and 2, that the pigs vaccinated with B.C.G. all showed moderately progressive tuberculosis while the unvaccinated controls all showed extensive tuberculosis.

It is interesting to note that the pigs vaccinated with the vole vaccine did not react to 1:1,000 dilution of O.T. while those vaccinated with B.C.G. were positive. The vaccinated animals, however, did react to lower dilutions of O.T. This work warrants further investigation.

IMMUNIZATION WITH KILLED BACTERIA.—While it is generally admitted that suspensions of living, attenuated organisms are more potent immunizing agents than suspensions of killed bacteria, the desire for safety has led to the widespread use of the latter. If the organisms used in its preparation are obtained from the patient, the vaccine is described as an "*autogenous*" one. When it is prepared from a stock culture it is said to be a "*stock vaccine*." If more than one kind of bacteria is included in the suspension, it is called a "*mixed vaccine*."

Since the term "vaccine" originally signified a preparation of virus from vaccinia or cowpox, it is evident that its use to desig-

nate an immunizing agent consisting of bacteria is not logical. Usage, however, has led to the policy of applying the term "vaccine" to practically all agents employed to produce *active immunity*. Agents used to produce passive immunity represent antibodies present in or obtained from blood serum and are therefore called *sera*, *antisera* or *antibody solutions*.

Bacterial vaccines are employed for many specific purposes which may be enumerated as follows:

1. Bacterial vaccines, especially typhoid vaccine, as well as sterile milk have been used in *nonspecific protein* therapy in such diseases as chronic gonorrheal rheumatism, various other types of arthritis, psoriasis and some other conditions. They are injected to produce a physiological reaction or protein shock in the patient.

2. *Specific vaccine therapy* has been employed in certain recurrent or chronic diseases such as acne vulgaris, gonorrheal arthritis, undulant fever, etc. The results reported are not very encouraging. Since bacteria have not been incriminated as the cause of the common cold one cannot regard the so-called "cold vaccines" as specific. It is interesting to note that when these "cold vaccines" have been tried out on a fairly large scale and under at least partly controlled conditions, the results have been disappointing.

3. *Specific vaccines* are injected into animals such as the horse, ox, goat and rabbit to *stimulate* the production of specific humoral *antibodies*. The animals are bled and the serum containing antibodies is separated from the cellular elements. In the commercial preparation of therapeutic and certain diagnostic sera the globulin fraction of the serum, containing antibodies, is precipitated, redissolved and concentrated. The most frequently used antibacterial sera are those for the various types of pneumococci, meningococci and streptococci. Antisera used to identify *E. typhosa*, the *Salmonellas*, various strains of dysentery and other bacteria are produced commercially, but they are frequently produced privately and used without being concentrated.

4. Bacterial vaccines are used quite extensively in *prophylactic immunizations* against the organisms causing typhoid fever and whooping cough. Typhoid fever is one of the preventable diseases that has decimated armies in the past. According to Jordan (1931) 60 per cent of the German mortality during the Franco-

Prussian War (1870-71) was due to typhoid fever. During the Spanish-American War, there was one case of typhoid to approximately every five men.

Largely through the efforts of Wright in England and Russell in the United States, prophylactic inoculation against typhoid and paratyphoid fever was introduced into the British and United States armies, respectively. It was made compulsory in the United States Army in 1911. It should also be added that excellent sanitary precautions have been introduced and enforced. The incidence of typhoid fever among the troops of the United States Army during the World War shows quite definitely the importance of these measures. There was only one case for every 3,756 men, which is in marked contrast to one for every five men during the Spanish-American War.

After the last war, according to Simmons (1941), the triple vaccine (against typhoid and paratyphosis A and B) was replaced first by typhoid-paratyphoid A and later by a typhoid vaccine only. Simmons states that the army has now gone back to the triple vaccine. He also mentions that pneumococcus polysaccharide solution is giving encouraging results in experimental immunization against pneumonia.

IMMUNIZATION WITH BACTERIAL POLYSACCHARIDES.—The employment by the army, as reported by Simmons, of pneumococcus polysaccharide is justified by the discovery of Francis and Tillett (1930) that Type I pneumococcus polysaccharide injected into human beings gave rise to mouse protecting antibodies. According to Horsfall and Goodner (1936) it has been shown that antibody response to the injection of Type I polysaccharide can be obtained in man, mouse, horse, cat and dog but not in the rabbit, rat, guinea pig and sheep. Boivin (1935, 1936, 1937) has apparently isolated carbohydrate-phosphatide compounds from *S. enteritidis* and *S. aertrycke* which stimulated the formation of specific antibodies in rabbits. Similar antigenic polysaccharide-phosphatide complexes have been isolated by Raistrick and Topley. The latter and his associates (1937) obtained antigenic fractions from *E. typhosa* which contained a large proportion of polysaccharide. It should be remembered, however, that not all type specific pneumococcus polysaccharides have been shown to stimulate

antibodies when freed from protein, yet all will react with antibodies to form precipitates, fix complement, etc. It is always difficult to exclude traces of protein in the polysaccharide preparation as important factors in antibody stimulation.

WHOOPING COUGH IMMUNIZATION.—While whooping cough immunization has been practiced for many years, it is only since Sauer (1933) published his results using vaccine composed of several freshly isolated smooth hemolytic strains of *H. pertussis* grown on human blood, that its value seems to be established by clinical experience.

Since Bradford and Slavin (1937) have reported the isolation of ten atypical strains of *H. pertussis* like organisms from cases presenting clinical evidence of whooping cough, it would suggest the existence of more than one serological type or strain of *H. pertussis*. These atypical strains have been called "*parapertussis*" since they darken the cough plate medium, show early pleomorphism and to a slight degree are said to resemble *B. bronchisepticus*. Sauer (1939) does not approve of combining them with *H. pertussis* in a vaccine as he thinks this would "weaken the vaccine."

Sauer's vaccine contains from 15 to 20 billion organisms per cubic centimeter. He recommends giving from 70 to 80 billion bacteria in divided weekly doses over a period of approximately 3 or 4 weeks. In his protocols he mentions giving young nonimmune children simultaneous injections of 10 to 15 billion in each arm for 3 successive weeks. In Sauer's hands, the results reported are very encouraging since he records failure of the vaccine to protect in only 1.3 per cent of 4,200 cases whereas the incidence in a control group was 13 times as great.

IMMUNIZATION WITH DETOXIFIED TOXINS.—According to Simons (1941) immunization with tetanus toxoid (detoxified toxin) is now employed by practically all armies. The United States Army attempts to establish an initial immunity to tetanus by administering three 1 c.c. doses of toxoid given three weeks apart. In order to maintain this immunity, an injection of 1 c.c. of toxoid is given at the end of one year, also at the time of departure for active duty in a theater of war if six months has elapsed since the last dose or whenever the soldier is wounded or exposed to tetanus infection.

In private practice, tetanus and diphtheria toxoids are employed quite extensively to immunize young children. Since diphtheria toxoid may give severe reactions in older individuals, it is customary to use toxin-antitoxin in individuals over 8 to 10 years of age. A more extensive discussion of immunization against diphtheria and other toxemic diseases is given in later chapters.

IMMUNIZATION PROGRAMS.—It is common practice among pediatricians and many general practitioners to follow a definite program of immunization against the common contagious diseases of childhood. While all physicians do not follow the same program, a fairly typical one is that suggested by Neff (1941). He advises that (1) "smallpox vaccination may be done at the time of birth, either on the delivery table or when coming home from the hospital, but there is sufficient immunity at that time so that the site of vaccination may not take and may have to be repeated at about three months of age. It is well in individual cases to make it a rule to vaccinate at three months of age and repeat each week if a take has not been obtained from the previous attempts. Vaccination against smallpox should be arranged for in any physical examination of a person who has never been successfully vaccinated. This holds true of all school children upon admission. It is well to inspect the site of vaccination two days following the application of the virus in order to see if there is a reaction of immunity. In such a case the explanation is furnished for a failure to take in the next few days. It is probable that more successful takes occur from using multiple sites at the time of vaccination.

(2) "Whooping cough vaccine may be given at any age in childhood but as a routine it may be planned for at the age of six months with weekly doses for the required number. There will be fewer reactions if the total amount of vaccine to be used in the individual case is divided up into five graduated doses. It is possible that by the use of pertussis antigen, beginning immediately after exposure of the child to a case of whooping cough, the child may be immunized successfully, or if unsuccessful that the disease when developed will be milder than would otherwise have happened.

(3) "Since most infants have relative diphtheria immunity until about nine months of age it is well to begin active immunization at that time, using three doses of regular diphtheria toxoid at three-week intervals or alum precipitate toxoid, three doses at two-

month intervals. Two months after either of these immunization programs the Schick test should be done as a routine in order to see that immunity has actually developed. In the infant who has actually received the immunization at that period of life and a Schick test found negative, the Schick test should be repeated once yearly until the age of five years to see that the immunity is being held. It is not rare for a child from two to five years to lose immunity, especially if he has had only one dose of alum toxoid, or two doses of regular toxoid. A check each year is of protective value.

(4) "Active immunization against tetanus may be obtained by the use of tetanus toxoid given at two-month intervals and may be combined with the diphtheria toxoid at the first and third doses of that toxoid. It may be prepared separately or combined with the diphtheria toxoid. It is well to use two such doses of toxoid following a passive immunization against tetanus with the tetanus antitoxin in which case, however, immunity is probably more complete. Therefore, whenever a protective dose of tetanus antitoxin is given in cases of an accident the individual should be given a dose of tetanus toxoid within two weeks and follow this two months later with a second tetanus toxoid.

(5) "Scarlet fever active immunization at the second or third year of age is advisable. Most children are susceptible at that age. One objection to the Dick toxin active immunization has been the reactions accompanying the rapidly increasing repeated doses recommended by Dick. This objection can be overcome by using half of the ampule at the injection, thus making ten doses rather than the regular five. There is practically no reaction from this attenuated dose. It is well to do a Dick test first and if negative it will be unnecessary to go ahead with immunization. The Dick test should be repeated two months after the series of injections so that one can see that there is a reversal of the previous positive test.

(6) "Measles Immune Globulin will produce passive immunity if injected within a day or two after exposure to measles. This should be done in frail, delicate, young children. But a modification of the disease may be obtained by one or two doses of this preparation given from six to eight days after a known exposure.

Fifteen c.c. of parental whole blood will accomplish much of the same effect given intramuscularly."

There are a number of reasons why such a program is desirable: (1) experience has shown that severe reactions are least likely to occur when immunization is carried out early in life; (2) it gives reasonable assurance of protection before exposure usually occurs; (3) it will probably lead to a larger proportion of the population being immune; (4) it spreads the injections of foreign protein out over a reasonable period of time.

The question of whether the child has developed immunity in response to vaccination often arises. There is usually little doubt as to whether the vaccination against smallpox is successful and the Schick test is a fairly accurate test for immunity to diphtheria. Many regard the Dick test as a good criterion for scarlet fever immunity, but this is much more in dispute than the results of the Schick test. In regard to immunity to tetanus, it is possible to determine the amount of antitoxin in the child's blood, but this involves too much time and expense to be made a routine test; hence it is assumed that immunity develops following the injection of tetanus toxoid. There is a great deal of clinical and laboratory data to support this assumption. There is, at present, no way of measuring a child's immunity to *H. pertussis* although his humoral (antibody) immunity can be measured. Clinical data, however, such as those reported by Sauer (1933) and others support the assumption that immunity results from the administration of his vaccine. It should be borne in mind that the local or systemic reaction to the injection of a bacterial vaccine is no index of either susceptibility or immunity.

The question is often asked as to why several instead of one injection of vaccine is given. There are probably several reasons for this. Dean and others seem to have shown that in developing humoral immunity, the first one or two injections are the *primary stimulus* and that to obtain best results, a *secondary stimulus* should be given about the time the titer reaches its peak, the object being to let some immunity develop before giving the secondary stimulus. With bacterial antigens the antibody titer usually rises for 7 to 10 days after an injection while the antibody titer following toxoid is much slower in reaching a peak. Experience has shown that a spacing of one to two months is de-

sirable, although for convenience shorter intervals are often used. After an immunity is once established, the question of how soon is it necessary to revaccinate arises.

In the case of smallpox, the Kansas City epidemic showed that no one successfully vaccinated within six months of exposure contracted the disease. Ordinarily it would seem wise to revaccinate whenever exposure occurs. If one is immune, the only reaction will be the immune reaction described by Jenner and this is not objectionable.

In the case of typhoid immunization, many advise revaccination every 2 years. With whooping cough and tetanus it is possible that immunity will last for several years although frequently revaccination may be indicated when exposure occurs. The time for revaccination against diphtheria can be determined by means of the Schick test.

An interesting phenomenon that may be of importance is the *anamnestic* reaction which is the basis for the timing of the secondary stimulus in vaccination. While it takes days or weeks for antibodies to develop following the one or more doses constituting the primary stimulus, after immunity is established, a secondary stimulus or injection of vaccine will lead to a rapid formation of antibody. It has been noted that in children whose Schick test was negative following immunization and a few years later became positive the amount of toxin used in the positive Schick test caused a rapid rise in their antitoxin titer so that in retesting they were negative. This is an example of the anamnestic reaction. It is because of this "hair-trigger" mechanism (anamnestic reaction) that revaccination following exposure is substituted. The revaccination dose is a secondary stimulus and causes a rapid rise in antibody titer. It is well established that the secondary stimulus does not have to be a large dose of antigen.

It should be remembered that, granting a good antigen and correct method of administering a vaccine, some individuals do not develop immunity. Furthermore, killed bacterial antigens (e.g., *E. typhosa* and *H. pertussis*) do not stimulate as great an immunity as results from the disease.

An explanation of this is, in part at least, to be found in the discoveries of Felix and Pitt (1934) and others studying the

antigenic components of *E. typhosa* and Mudd, Pettit, and Lackman and Morgan (1939) studying the antigenic components of virulent streptococci. This work has been mentioned under virulence in Chapter I. It will be recalled that virulence is associated with capsule formation and that each kind of bacterial cell is made up of a number of antigenic components, any one of which may give rise to antibodies, depending perhaps upon its location in the cell.

It has been emphasized by Landsteiner, Nicolle, Zinsser and others that there is a "mosaic" of antigens in a bacterial cell. The position and the way the antigens are oriented relative to the surface are important factors. The surface antigens may act as a barrier to antigenic components beneath the surface. These are important concepts when one is considering the factors that determine the immunizing value of a vaccine. It is known, e.g., that *E. typhosa* can dissociate into smooth motile, smooth nonmotile, rough motile and rough nonmotile variants.

It was thought, until 1934, that the best typhoid vaccine should consist of *any* smooth motile strain because it would contain O (somatic) and H (flagellar) antigens. Felix and Pitt, however, discovered that an additional antigenic factor determining virulence is important. They named this factor the "Vi" antigen.* It is apparently a surface antigen and is destroyed by heat and many chemicals. More recently Mudd and his associates (1938, 1939) have shown that virulent streptococci contain a partial heat labile antigen obtained by physical means of disintegration. It is probable that many and perhaps all virulent bacteria contain labile antigens important in immunization. It is perhaps because of the heat lability of these antigens and the ease with which they are destroyed by most chemicals that killed bacteria are not as good immunizing agents as virulent living ones.

Apparently formaldehyde can be used to kill bacteria with only partial destruction of the "Vi" antigen. This may be the reason formalized vaccines have been reported as superior to heat killed ones.

PASSIVE IMMUNITY.—When a serum containing antibodies is injected into a patient, the latter is being passively immunized. His own tissue cells did not produce these antibodies. In other words,

*Topley and associates have isolated two carbohydrate-lipid complexes corresponding to O and Vi antigens respectively, *Lancet* 232: 252, 1937.

the patient is passively accepting antibodies produced by another animal. The administration of antitoxin to prevent either tetanus or diphtheria is conferring passive immunity to the recipient.

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CHAPTER VII

IMMUNITY MECHANISMS IN EXPERIMENTAL INFECTIONS

The question of resistance to infection has been studied experimentally with considerable profit since Ashoff and Maximow crystallized our present concept of the reticulo-endothelial system. This chapter will be devoted to summarizing results and conclusions of Gay, Cannon, Teale, Goodpasture and others who have investigated the mechanism of resistance employed by the animal body to localize infection or to free the blood stream of bacteria and to favor recovery from infection.

Experimental Streptococcus Infections in Rabbits.—Gay (1923, 1926) and his colleagues studied the defensive mechanism of the pleural cavity of rabbits against experimentally produced infection with a highly virulent hemolytic streptococcus. They first injected a sterile irritant (aleuronat) which caused an inflammatory reaction in the pleura with the formation of granulation tissue. Polymorphonuclear leucocytes appeared in great numbers early in the reaction. Later there was an increase in mononuclear phagocytes both in the exudate and within the tissues.

If virulent streptococci were introduced when the neutrophils predominated, the animals died as rapidly as the controls. If they were injected after the mononuclear phagocytes were mobilized, the animals could withstand many lethal doses of streptococci inoculated either into the pleural cavity on the side where granulation tissue had been produced or into the opposite side where none was present. In the latter case the protection was due to clasmatoocytes coming over as reinforcements from the depot of mobilization in the opposite side. Gay and his associates speak of this as “*transpleural mobilization*” of clasmatoocytes.

Linton (1928), working in Gay's laboratory, *transplanted irritated omentum* (rich in clasmatoocytes) into the peritoneal cavity of normal rabbits and found that the rabbit's resistance to intrapleural infection with *Streptococcus hemolyticus* was greatly increased.

IMPORTANCE OF ANTIBODIES.—More recently Gay and Clark (1930)* have offered experimental evidence indicating that antibodies enhance the cellular defensive mechanism. In their opinion the reason that antibacterial, e.g., antistreptococcal immune serum, fails to combat a streptococcus infection is not a lack of antibodies but an absence of mobilization of elasmatoocytes in the patient. They found that "in the case of experimental streptococcus empyema in the rabbit the course of the ordinary fatal infection is in no wise affected by the transfer of the pleural fluid containing large numbers of mononuclear cells derived from an animal that is protected as a result of nonspecific irritation. The serum of a rabbit highly immunized against the streptococcus and containing antibodies for it, produces relatively slight effect in prevention or cure."

PASSIVE IMMUNIZATION WITH PLEURAL EXUDATE.—"In contrast to this the pleural exudate, either acute (polymorphonuclear) or subacute (mononuclear) produced in an actively immunized animal does protect passively to a considerable degree. In a similar fashion normal exudate cells of either type in combination with the relatively ineffective antiserum give a high degree of protection." In other studies Gay and Oram (1931) report that elasmatoocytes are much more resistant to *Streptococcus leucocidin* than the neutrophils. They regard this as further evidence of the superiority of "tissue macrophages" over neutrophils in combating streptococcus infections.

Experimental Staphylococcus Infections in Guinea Pigs.—In regard to tissue resistance to staphylococcus infections Freedlander and Toomey (1928) have studied *nonspecific mechanisms* and Cannon et al. *specific factors* in the skin of guinea pigs. The former compared the inflammatory response in the subcutaneous tissues of normal guinea pigs with the response in guinea pigs whose skins had been treated with nonspecific irritants. They observed that the latter were protected for short periods of time by tissue macrophages mobilized by nonspecific irritation.

Cannon et al. (1930, 1932) have investigated tissue immunity to staphylococcus infection in normal and vaccinated guinea pigs. They find that when staphylococci are injected into the skin of the abdominal wall of normal guinea pigs there results an inflammation

*Gay, F. P., and Clark, A. R.: J. Exper. Med. 52: 94, 1930.

characterized by an infiltration of neutrophils which actively phagocytize the bacteria. This process does not lead to a localization of the infection, but instead, the latter spreads throughout the subcutaneous tissue in the form of a cellulitis.

On the other hand, when they inject staphylococci into the skin of previously immunized pigs the organisms are agglutinated into masses of various sizes, and there is an infiltration of cells from the subreticular layer of the cutis where vast numbers of "tissue macrophages" have accumulated as a result of the previous intracutaneous vaccination. Many of the clumps of bacteria are phagocytized by the large mononuclear phagocytes and the infection remains localized because of phagocytosis and other factors.

LOCAL FIXATION.—In discussing their results Cannon and Pacheco (1930) call attention to the significant work of Opie (1929) which enabled them to offer a rational explanation of the observed phenomena. Opie studied the Arthus phenomenon and concluded that when one injects an antigen into tissues containing antibodies the resultant antigen-antibody complex acts as a tissue irritant, with a resulting inflammatory reaction characterized by an infiltration of neutrophils, edema, and the deposition of fibrin. The small blood and lymph vessels are injured and become thrombosed. He obtained similar results when he injected antigen and antibody simultaneously into the tissues of the rabbit. Of considerable significance is his observation that antigen injected into the skin of normal rabbits passes rapidly into the blood whereas antigen injected into immune animals is fixed at the point of injection. When along with these results one takes into consideration the work on opsonification discussed in Chapter V, it would appear that Cannon's explanation of tissue immunity to staphylococci observed in the immunized guinea pigs may be summarized as follows:

SUMMARY OF CANNON'S WORK ON TISSUE IMMUNITY TO STAPHYLOCOCCI.—

1. As a result of intracutaneous vaccination there is a mobilization of mononuclear phagocytes within the subreticular layer of the cutis.
2. There is an accumulation of antibody (opsonins, agglutinins, etc.) within the tissues which is perhaps adsorbed by tissue cells.

Since in Cannon's opinion the antibodies are produced largely by the reticulo-endothelium, it is probable that antibody is also contained within the tissue macrophages.

3. When virulent staphylococci are injected after the guinea pigs have become sensitized, the bacteria adsorb antibody, there is an increase in the cohesive (sticky) property of their surface and as a result of physical-chemical changes many of them agglutinate. This antigen-antibody complex should lead to anaphylactic inflammation.

4. The filming of bacteria by antibody with resulting surface changes constitutes the process of opsonification which favors phagocytosis.

5. The bacteria are phagocytized and destroyed within the food vacuoles of the clasmatoocytes. The latter contain antibody which means that they may be affected by antigen-antibody reactions. This may be a factor in the development of an intense secondary inflammation accompanied by an increase of neutrophils and clasmatoocytes.

The organisms remain localized not only as a result of the edema and thrombosis of blood and lymph vessels but also because of the factors just mentioned.

Immunity to Pneumococcus Infection.—There is more or less controversy in regard to the defensive mechanism against pneumococcus infection. Gay and Clark (1930) say that while mononuclear mobilization by *nonspecific* irritation is effective against streptococci, it is of little value in pneumococcus infection unless the pneumococci are first treated with a specific immune serum. Apparently when the pneumococci are opsonized the mobilized mononuclear defense is effective. Nakahara has also investigated the effectiveness of clasmatoocytes mobilized in the walls of the peritoneum of mice by the intraperitoneal injection of olive oil. He found increased resistance to staphylococci, pneumococci and *B. coli*.

Cannon and his associates, Walsh and Hartley (1936, 1938), have studied upper respiratory tract infections in normal, immune and allergic rabbits. They have also investigated the effect of various substances used in intranasal medication upon the de-

fensive mechanisms of the respiratory tract. The results of their work may be summarized as follows:

1. By intranasal vaccination with a formalin-killed culture of pneumococcus Type I or with an autolysate they were able to confer practically complete protection against living pneumococci introduced intranasally.

2. Such nonspecific irritants as formalin, tannic acid, alum or a paratyphoid vaccine used as intranasal stimulants did not confer protection against intranasal infection with pneumococci.

3. They found *intranasal* immunization *superior* to *intragastric* immunization with a pneumococcal vaccine.

4. They found no correlation between antibody titer in the blood and intranasal immunity since intranasally vaccinated rabbits, having no demonstrable antibodies in their blood, resisted intranasal infection.

5. Successful intranasal vaccination did not prevent the aspiration of intranasally instilled pneumococci into the lungs. It did *modify* their passage from the lungs to the blood possibly because of a general immunity and perhaps because of a locally enhanced capacity of the lungs to inhibit growth and spread of the pneumococci reaching them.

6. They concluded that when rabbits are kept in a normal position the virulent pneumococci instilled intranasally do not pass through the epithelium of the upper respiratory tract but are aspirated into the lungs and then enter the blood.

7. They confirmed the work of Freund (1927) and Freund and Whitney (1928) that, following the development of general active or passive immunity, the antibodies are found rather uniformly distributed throughout the tissues and the ratio of tissue antibody to serum antibody is usually between 1:10 and 1:15. This is called the T:S ratio.

8. When animals were vaccinated regionally as by intranasal instillation the regional tissue (mucous membrane) serum ratios of antibody was on an average of one to five. Cannon et al. state that this suggests that perhaps the antibodies are formed locally in regionally stimulated tissues and diffuse into the blood.

9. Cannon and Walsh (1938) followed up the work mentioned above by studying the effect of many substances used in intranasal medication on the local defensive mechanisms. Their work

is of special interest in view of the increase in the number of cases of lipid pneumonia reported in infants as resulting from intra-nasal medication.

Cannon and Walsh found that mineral oil, which is a popular vehicle for menthol, eucalyptol, iodine, guaiacol, etc., readily reaches the lungs when instilled into the nose of rabbits. They point out that it is quite liable to pass from the nose to the trachea and lungs of infants lying on their backs. This may account for much of the lipid aspiration which is found at autopsy. The mineral oil and many other fluids used for nasal medication such as argyrol, etc., can carry bacteria from an infected nose or nasopharynx to the lungs.

Cannon and Walsh also cite other research that indicates that many substances used in intranasal medication interfere with the ciliary movement or by other means interfere with the streaming of mucus over the mucous membrane and thus paralyze a protective mechanism. Not only do mineral oil and some other substances affect the cilia but when they reach the lungs they affect the permeability of the blood capillaries. They state, however, that some materials such as prontosil, 1 per cent thymol, 1 per cent menthol, 10 per cent glycerin or any of the solutions of the vaso-constrictors in isotonic saline are not likely to be injurious although some of them do affect the cilia.

10. Cannon and Hartley sensitized rabbits to egg albumin and then injected a mixture of pneumococci and egg albumin locally to see if the allergic inflammation would protect against the pneumococcus. As a result of this and a number of other similar experiments they conclude that allergic inflammation *failed* to protect rabbits against infection with virulent pneumococci. These findings are in harmony with those of Rich (1933). He mixed a small dose of fowl cholera bacilli with pneumococcus polysaccharide and injected the mixture into a rabbit sensitive to the polysaccharide. The resulting allergic inflammation did not protect the rabbit since a generalized infection ensued.

Experimental Infection of the Chorio-allantoic Membrane of Chick Embryos was carried out by Goodpasture and Anderson (1937) with results that appear to throw light upon susceptibility as well as resistance. Goodpasture's timely observation, that while

resistance has been investigated extensively the problem of *susceptibility mechanisms* has been neglected, should stimulate research of great value to scientific medicine. The results of their experimental studies may be summarized as follows:

1. They suggest that the inoculation of the chorio-allantoic membrane of the chick embryos with pure cultures of bacteria is an excellent method for studying the early stages of invasion.

2. They produced experimental infections with *Staph. aureus*, *Str. haemolyticus*, *Str. viridans*, and *A. aerogenes*, *E. typhosa*, *Br. abortus*, *C. diphtheriae*, and *Myco. tuberculosis avium*.

3. They found that either mesodermal cells (fixed or motile), or epithelial cells, or both, seem to act as host-cells for all of the organisms mentioned except *Staph. aureus*, *Str. haemolyticus* and *C. diphtheriae*. *Str. viridans* utilized the intracellular environment of wandering and fixed mesodermal cells; *A. aerogenes* the wandering mesodermal and endodermal cells; *E. typhosa* utilized cells of the ectodermal epithelium; *Br. abortus* utilized both ectodermal and mesodermal cells while *Myco. tuberculosis avium* preferred mesodermal elements.

The staphylococcus and hemolytic streptococcus grew well in between the tissue cells and underwent phagocytosis but were apparently unable to utilize any living intracellular environment for growth.

4. These results caused Goodpasture (1937) to investigate carefully the pathogenesis of typhoid fever. As a result of his study of excellent autopsy material he found *young* plasma cells in sections of the lymphoid follicles of iliac and mesenteric lesions filled with small, gram-negative apparently unaltered bacilli which he judged to be *E. typhosa*. He found larger gram-negative bacilli in macrophages of the lesions associated with remnants of lymphocytes and macrophages. Goodpasture suggests that the *young* plasma cell is a host for *E. typhosa* in human cases and nourishes and protects the bacteria during the incubation period and throughout the disease.

Rôle of Clasmatocytes in Other Infections.—That the reticulo-endothelial system is an important factor in combating both bacterial and protozoan infection is suggested by the results of a number of investigators. Buxton (1907) concluded that the resistance of rabbits immunized against typhoid is due largely to

the increased ability of the mononuclear phagocytes to engulf and destroy typhoid bacteria. Cannon and Taliaferro (1931) carried out an extensive investigation of the cellular reactions in the tissues of canaries infected for the first time with *Plasmodium cathemerium* and also in superinfected birds. This last observation indicates that the primary infection alters the reactivity of the mesenchyme so that phagocytosis is more rapid when a secondary infection is produced.

In 1936 Taliaferro and Cannon reported upon the cellular reactions during primary infections and superinfections of *Plasmodium brasilianum* in Panamanian monkeys. They say that the macrophages of the spleen, liver and bone marrow are the primary defense against malaria. They suggest that the reason for this is probably the slow circulation of the blood through these tissues which allows for direct contact of the blood and macrophages whereas elsewhere in the body similar conditions do not exist.

TISSUE RESISTANCE TO CYSTICERCUS PISIFORMIS.—In a study of experimental infections of *Cysticercus pisiformis*, a larval tapeworm, in passively immunized rabbits, Leonard (1940) showed that host resistance is expressed parenterally as an enhanced and accelerated tissue response; larvae in the liver were killed in these animals in approximately one-half the time required for their death in normal animals. Leonard and Leonard (1941) found that in passively immunized rabbits, the intestinal wall, or some substance released by the host tissues within the intestine, plays a major part in the total resistance mechanism. This was shown by injecting artificially hatched larvae into the mesenteric veins, thus allowing them to reach the liver without penetrating the intestine. It was shown by this means that, in passively immunized animals, only 3 to 4 per cent of the larvae are capable of overcoming the resistance of the host intestine, while in normal animals, 85 per cent of the larvae are able to penetrate the intestine and safely reach the liver.

Removal of Bacteria from the Blood Stream.—Cannon, Sullivan and Neckermann* (1932) investigated the conditions in-

*Cannon, P. R., Sullivan, F. L., and Neckermann, E. F.: J. Exper. Med., 55: 121, 1932.

fluencing the disappearance of living bacteria from the blood stream. They interpret the general mechanism as follows: "Staphylococci injected intravenously into normal rabbits circulate throughout the blood stream in large numbers, probably making many passages through the organs and tissues of the body. In passing through the spleen and liver, especially, conditions more favorable for phagocytosis may obtain, particularly those dependent upon slow blood flow, availability of macrophages and leucocytes, mechanical conditions favoring filtration, etc. Chance contacts between phagocytic cells and the relatively unchanged staphylococci induce a certain degree of phagocytosis, as seen in the polymorphonuclear leucocytes in the lungs and in the macrophages and leucocytes of the liver and spleen. Eventually this mechanism removes the bacteria from the circulating blood. Their further fate doubtless depends upon the virulence of the microorganisms and the digestive capacities of the macrophages or in other words the functional state of the phagocytes, both macrophages and polymorphonuclear-leucocytes. As Werigo showed in experimental anthrax infection, if these cells become inadequate, the bacteria again multiply and generalize.

"In the immune animals, this normal mechanism disposed of the dead bacterial bodies during the preliminary period of immunization, at which time many macrophages in the liver and spleen removed and digested the bacterial particles. When at a later period large numbers of living staphylococci run the gauntlet of those macrophages, there is an almost instantaneous swelling of the *micro-organisms*, an increasing obstruction to their free passage through the immune liver and spleen and a tendency to clumping of the bacteria with a resulting *retention* of such affected microorganisms in these organs."

In regard to the relative importance of macrophages and neutrophils Cannon, Sullivan and Neckermann, state that "in such a complex system, however, it is questionable whether too much emphasis should be placed upon the comparative significance of two groups of mesenchymal cells whose functions are so similar and apparently complementary." They observed phagocytosis by both groups of cells. They do state, however, that the "primary reaction is mainly between the cocci, immune bodies and the cytoplasmic surfaces of the macrophages, accompanied or quickly followed

by the accumulation of polymorphonuclear leucocytes attracted or retained there by chemotactic or electrotrophic influences." Both types of cells engulfed and destroyed bacteria, the outcome of the battle depending upon the virulence and number of the latter.

Teale (1935) reports the results of an extensive study of the relative importance of the reticulo-endothelial tissues and the circulating antibody in immunity. He reports that immunized animals whose blood did not contain agglutinin, germicidal power or protective antibody tested in passive transfer, were able to clear completely the peripheral circulation of highly virulent bacteria against which they had been immunized.

He also gives protocols showing that normal animals, which he *assumed* had no circulating antibodies, were likewise able to free the peripheral circulation of virulent organisms although they could not prevent the occurrence of secondary waves of fatal bacteremia as could the immune animals.

These results are not out of line with the findings of others and do not, in the case of immune animals, exclude antibodies present in the tissues from playing a rôle in defense. Furthermore, in the immune animals the anamnestic reaction (hair-trigger mechanism) might operate and circulating antibodies appear following the test injection of bacteria.

In regard to his protocols on normal animals he states that "the rabbit is generally stated to have *no* antibody against *B. dysenteriae* (Shiga) nor the organism of fowl cholera." He has apparently accepted this assumption in lieu of testing the rabbits he used. There seems to be good reason to question this assumption since Mackie and Finkelstein (1931, 1932) reported that normal rabbit blood is frequently germicidal for *B. dysenteriae* (Shiga), and Coriell, Miller, and Sherwood (1940) confirmed the conclusion of Mackie and Finkelstein and in addition found bactericidal antibodies for the organism of fowl cholera. Teale apparently assumes that such antibodies do not exist.

In spite of these differences of opinion we are in agreement with his major thesis that resistance to infection does not necessarily parallel the content of *circulating* antibody. In fact we have cited other literature to that effect and also work done in this laboratory showing that no virucidal antibody could be demonstrated in cats exhibiting a solid immunity to a strain of vaccine virus that pro-

duced fatal infection in rabbits. We are, however, convinced that the presence of antibodies intimately associated with the tissue cells is of prime importance as an adjunct to cells of the reticulo-endothelial system in resistance. In immune animals the anamnestic reaction may lead to an increase of tissue antibodies even though they are not in detectable amounts in the blood.

Defensive Mechanisms in Peritonitis.—One of the pathological states in the human that frequently results in generalized infection and an overwhelming toxemia is peritonitis. Hertzler's (1919) extensive and very valuable study of the anatomy, physiology, pathology and defensive mechanism of the peritoneum deserves every medical student's attention. He found by experiment that isotonic solutions and particulate matter injected into the normal peritoneal cavity were rapidly absorbed directly into the blood, although he does not deny that absorption by way of the lymphatics occurs. Absorption is delayed when the intraabdominal pressure is sufficient to interfere with venous flow. *Tympanitis* may therefore be of value in retarding absorption. He calls attention to the possibility of increasing absorption from the peritoneal cavity in such cases when the intraabdominal pressure is reduced too rapidly. If he produced an inflammatory exudate of the peritoneum before injecting fluid or particulate matter into the cavity, absorption was retarded or prevented.

He discusses the method of formation and the importance of *temporary adhesions* in limiting the spread of infection within the abdomen. In regard to the nature of the cellular response in peritonitis he states that neutrophils predominate for a period, and if the infection is being successfully checked, mononuclear cells make their appearance. On the other hand, "when patches of viable and degenerated leucocytes coexist, an advancing condition may be assumed."

Altmeier and Jones* (1940) report an interesting comparison of postoperative results in fifty-one consecutive cases of resection for carcinoma of the rectum and sigmoid reported by Pratt and a series of experiments on experimental peritonitis in rabbits which they conducted. It was noted that the absence of postoperative peritonitis in the cases reported by Pratt apparently correlated with preoperative high voltage roentgen therapy one month to six weeks previous to operation. An investigation of the effect of such

*Altmeier, W. A., and Jones, H. C.: Experimental Peritonitis, J. A. M. A. 114: 27, 1940.

treatment upon rabbits carried out by Altmeier and Jones showed that preoperative high voltage roentgen irradiation was of value in developing immunity against experimental peritonitis. They offer no theory of the mechanism involved.

Defense Against Viruses.—In regard to the defensive mechanism against viruses, the prevailing opinion as expressed by Rivers (1928) and Aycock (1931) seems to be that it is both cellular and virucidal or humoral in nature and that the inflammatory response is secondary to cell injury. This is apparently borne out by the experimental work of Andrews (1930). Some of his conclusions are summarized as follows: (1) The virus of herpes can be cultivated successfully in tissue cultures (rabbit testes) if dilute rabbit serum is employed. (2) Neither growth of the virus nor the formation of inclusion bodies is obtained in tissue cultures if immune serum is added to the culture before the virus or together with it. (3) The virus of herpes will grow and form inclusion bodies in tissue cultures when immune rabbit testes and normal serum are used. (4) Normal tissue is infected by the virus within one-half hour at 17.5° C. or 37° C. if immune serum is not present; the later addition of immune serum does not prevent growth of the virus nor the formation of inclusions.

Jamuni and Holden (1934) present evidence which indicates that both leucocytes and immune serum play a rôle in immunity against herpes virus. The mononuclear cells are apparently more efficacious than polymorphonuclear cells in disposing of herpes virus. They suggest that the virus is opsonized and then phagocytized although they were unable to verify this point. Their results indicate that phagocytic cells (immune or normal) bring about a greater virucidal effect than can be obtained with immune serum alone.

Tolerance.—In addition to the various factors of defense discussed in this chapter the phenomenon of "tolerance" perhaps should be mentioned. Gunn (1923) regards this as very important especially as regards certain toxemic diseases. He cites the *specific* congenital tolerance of the toad, rat and grass snake to toad poison which has a digitalis-like toxic action and is a glucoside. He says that these same animals show a high degree of tolerance to members of the same group of glucosides. The resistance is due to an insusceptibility of tissue, especially that of

the heart, to these glucosides. This is of interest in view of the resistance of the rat to diphtheria toxin. This has been studied extensively by Coea and others. The toxin apparently circulates in the blood and does not combine with the tissues. Further work is apparently necessary before such a phenomenon can be designated as a significant factor in *active immunity* in man.

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CHAPTER VIII

NATURAL AND IMMUNE ANTIBODIES

During the decade between 1880 and 1890 two theories arose as to the nature of the body's defense against infectious agents. The one, under the leadership of Flüggé and von Fodor in Germany and Nuttall in England, conceived of the body's defense as being due to chemical substances in the blood, while the other, under the leadership of Metchnikoff, maintained that when infectious agents entered the body, they were engulfed (phagocytized) by certain wandering and fixed tissue cells and ultimately destroyed by a process of digestion. The former is known as the humoral theory, while the latter is the cellular theory of immunity.

The Humoral Theory of Immunity, according to Ledingham, had its origin in Lord Lister's (1880-81) experiments on the keeping quality of aseptically removed ox blood. Blood itself is a complex circulating body fluid which functions as a carrier of oxygen and of the nutritive, waste and other materials of the body's metabolism. About 30 to 40 per cent of it is made up of cellular material while the remainder is the fluid portion or plasma. This latter contains 90 to 92 per cent water and 8 to 10 per cent proteins, carbohydrates, fats, electrolytes and a wide variety of substances of unknown structure such as enzymes, antienzymes, antibodies, etc. When blood clots, there is squeezed out a straw-colored fluid called *serum* which differs from plasma in that the fibrinogen has been removed in the process of clotting. Blood from which fibrinogen has been removed is called defibrinated blood.

Locke and Hirsch* state (1928) that chemical concepts of immunity had become sufficiently crystallized by 1887 to lead Emmerich to make the following suggestion: "It should be an important task for investigators to seek out the substances which are associated with the immunity state and to ascertain in what chemical grouping they belong."

*"The Isolation of Substances with Immune Properties," by A. Locke and E. F. Hirsch, in *The Newer Knowledge of Bacteriology and Immunology*, edited by E. O. Jordan and I. S. Falk. Reprinted by permission of the University of Chicago Press.

Alpha Lysins.—Between the years of 1888 and 1890, Nuttall in England and von Fodor on the Continent published their investigations as to the germicidal property of blood. By mixing defibrinated blood with bacteria and plating out after a period of incubation, they were able to show that *some* defibrinated bloods possessed mild germicidal properties. This seemed to be lessened or destroyed by heating to 60° C. for a short time or by standing for a longer time at room temperature.

These observations were confirmed by Buchner (1889) who regarded the germicidal property of blood as due to an unstable, thermolabile substance which he called *alexin*. By animal inoculation experiments, he showed that bacteria were destroyed by humoral elements in serous cavities of the body and in the subcutaneous tissues. Buchner conceived of resistance as due to *alexin*. This thermolabile bactericidal substance is also called *alpha lysin*.

IMMUNE LYSINS AND PFEIFFER'S PHENOMENA.—In 1894 Pfeiffer was studying cholera immunity and noted that when those guinea pigs which recovered from infection with *Microspira comma* were reinoculated intraperitoneally, the bacteria were rapidly destroyed and the animals survived, whereas in normal pigs very little destruction of the organisms occurred and the animals succumbed. Pfeiffer made three other observations that are fundamental to our present concepts. He found *first*, that the immunity enjoyed by the recovered pigs was *specific* for cholera vibrios and not for other bacteria; *second*, he could convey this immunity to normal animals by injecting intraperitoneally a small amount of blood from immune animals together with the virulent vibrios; *third*, Pfeiffer found that *heated* immune serum when injected together with the vibrios was effective in protecting the normal pigs. This was quite puzzling since he had previously confirmed Buchner's conclusions that in test tube and plating experiments, heated serum or defibrinated blood has apparently lost its germicidal property. Pfeiffer decided that some substance from the endothelial cells lining the peritoneum was able to restore to heated serum its germicidal property.

PFEIFFER'S IMMUNITY UNIT.—It is interesting to note that Pfeiffer realized the importance of devising some method for

measuring the strength of immune serum. This he did by selecting a number of guinea pigs of uniform weight and inoculating a series with varying amounts of heated immune serum mixed with two milligrams of virulent bacteria, an amount sufficient to insure death in normal guinea pigs when inoculated alone. The least amount of immune serum that dissolved the bacteria and protected the animal was called an "immunity unit."

BORDET'S EXPLANATION OF LYTIC MECHANISM.—It remained, however, for Jules Bordet (1898) working in Pasteur's laboratory to investigate carefully and explain the phenomena described by Pfeiffer. His results may be briefly summarized as follows:

1. He confirmed Pfeiffer's observation that normal guinea pigs could be protected by injecting them with immune serum. This is now called *passive immunity* as contrasted with *active immunity* enjoyed by animals that recover from an infection.

2. He further showed that fresh unheated serum from cholera immune guinea pigs would specifically kill and dissolve cholera vibrios in a hanging drop preparation under the microscope. He found normal guinea pig serum to be only slightly potent and not very specific.

3. In addition, he observed that the serum from immune guinea pigs, when heated to 55° C. for one-half hour and mixed with vibrios in hanging drop preparations, would frequently clump the organisms (agglutinate them), but would not kill or dissolve them. However, if he added a little fresh normal serum, which by itself had no germicidal properties, to the hanging drop of bacteria and immune serum, the vibrios were quickly killed and dissolved. Pfeiffer had been unable to render heated immune serum potent, but Bordet definitely proved it possible. Thus Bordet showed that fresh serum from nonimmune as well as immune animals contains a thermolabile or heat sensitive substance (called *complement* by Ehrlich) which will act upon and destroy bacteria suspended in heated immune serum.

Bordet concluded that some specific thermostable (heat resistant) substance in the immune serum rendered the cholera vibrios susceptible to the digestive action of complement. The chemical substance in the immune serum that renders bacteria sensitive to complement has been called by various names such as protective

substance (body protector), immune body (Pfeiffer), *sensitizer* (Bordet), and *amboceptor* (Ehrlich). Bordet showed that if he vaccinated guinea pigs with a killed vaccine, the protective substance or sensitizer appeared in their blood after the lapse of several days and that it was the same as the sensitizer found in the blood of animals recovering from infection.

It is now possible to develop these simple definitions:

1. **BACTERIAL AMBOCEPTOR, IMMUNE BODY OR SENSITIZER.**—This is a thermostable substance found frequently in small amounts in the blood of normal animals but more frequently and in much greater concentration in the blood of vaccinated animals or animals which have recovered from an infection. It has a specific affinity for the bacteria used in the vaccine or which caused the infection and by combining with them renders them susceptible to union with a normal enzyme-like substance called complement which kills and in the case of cholera vibrios dissolves the bacteria.

2. **SENSITIZED CELLS.**—When bacteria or any cellular antigen is united to its antibody or sensitizer it is then a sensitized cell.

3. **COMPLEMENT.**—This is a thermolabile enzyme-like substance found in the blood of all warm-blooded and some cold-blooded animals. It will combine with sensitized cells and bring about their death or lysis.

Further Studies on Normal Bacteriolytic Substances.—In addition to the *alpha lysins* which require thermolabile complement for their activity other lytic agents such as *beta lysins*, *leukins* and *lysozyme* have been described. Each of these would seem to merit a brief discussion.

ALPHA LYSINS.—The question arose quite early as to whether the lytic mechanism described by Bordet for immune lysins would apply to normal lytic agents. Muir and Browning (1908) working with guinea pig serum, concluded that the mechanism of normal bactericidal action was similar to the bactericidal action of immune serum. They further concluded that bactericidal complement (termed by them "bacteriophilic" complement) and hemolytic complement are separated varieties. Other research such as that of Irwin, Beach and Bell (1936), or Shrigley and Irwin (1937), and of Dingle, Fothergill and Chandler (1938) tends to substantiate this conclusion. Mackie and Finkelstein (1931, 1932) carried out

an extensive investigation of thermolabile bacteriolysins. They concluded that the bacteriolysins are widely distributed in the animal kingdom, are specific for the bacteria they act upon and that they consist of thermostable antibody (amboceptor) and thermolabile complement. In their opinion the antibodies, developed as a result of either vaccination or infection, have their precursors specifically differentiated in the serum of normal animals and that, in general, they are not substances formed *de novo*. Coriell, Miller, and Sherwood (1940), working in this laboratory, confirmed many of the conclusions and extended the experimental findings of Mackie and Finkelstein. They found the serum of 20-hour-old kittens strongly bactericidal; the serum of 3-day-old rabbits moderately bactericidal; and the serum of very young chicks and chick embryos weakly bactericidal. Since these bactericidal substances were inactivated at 56° C. and could be specifically absorbed they were apparently α lysins.

β LYSINS, LEUKINS AND LYSOZYME.—These are bactericidal substances having many properties in common with each other but distinctly different from α lysins. While α lysins are quite active against typhoid-colon, *Salmonella* and dysentery groups as well as *V. cholerae*, *B. pyocyaneus*, pneumococcus and streptococcus, the β lysin and leukins are active against anthrax-subtilus and proteus groups. Ledingham (1931) gives an excellent brief discussion of these substances. It appears that their titer is not increased by vaccination and they are more thermostable than the α lysins. The β lysins are found in normal sera, the leukins are present in leucocytes, while lysozyme is present in various secretions and tissues of the body. It is especially present in tears and cartilage. Lysozyme is most active against a saprophytic micrococcus isolated by Fleming. While β lysins are more thermostable than α lysins they apparently are of complex nature. Pettersson (1928, 1929) says that the portion analogous to amboceptor is denoted the "activable substance" and that corresponding to complement the "activating substance," which is destroyed at temperatures between 63° and 70° C. Apparently the activable substance does not combine with the bacteria unless the activating substance is present. It is still unknown what role these substances play in the body's defense.

Natural and Immune Bacterial Agglutinins.—The capacity of normal or immune serum to bring about the specific clumping (agglutination) of bacteria depends upon its antibody content. The titer of normal agglutinins varies from zero to one in ten or one in twenty; rarely is it found higher. On the other hand the titer of agglutinin following vaccination or recovery from infection is usually quite high in comparison. This subject will be discussed in a later chapter.

Hemolysins, Hemagglutinins and Other Antibodies.—While Bordet was busily engaged in explaining the mechanisms of bacteriolysis by immune serum, an important observation of Buchner attracted his attention. The latter had observed that occasionally normal blood serum had the property of destroying and hemolyzing foreign red blood cells in vitro (outside the body). Bordet wondered whether this phenomenon was dependent upon a sensitizer or amboceptor and complement mechanism such as he had shown for bacteriolysis and whether he could make specific sensitizer or amboceptor appear in the blood stream of animals by vaccinating them with a suspension of foreign red blood cells.

By a series of simple experiments he showed that in a normal rabbit whose blood serum had little if any effect upon sheep red cells, he could, by injecting it several times with washed sheep cells, cause the appearance in the blood stream of the rabbit, within about a week after the last injection, of an appreciable amount of sensitizer or hemolysin for sheep cells. He found that this new sensitizer or hemolytic amboceptor for red cells was thermolabile, that it did not possess the property of destroying the cells, but, like bacteriolytic sensitizer, it rendered the red cells susceptible or sensitive to the digestive or lytic action of complement. This resulted in the liberation of hemoglobin from the red cells, a phenomenon which is called *hemolysis*.

The natural antibodies causing hemolysis and agglutination of foreign red cells are called *heterohemolysins* and *heterohemagglutinins*, respectively, while similar antibodies that result from vaccinating with foreign red cells are called *immune hemolysins* and *immune hemagglutinins*, respectively.

Landsteiner observed early in this century that human blood could be arranged into several groups, depending upon the property of human blood serum from one person clumping and fre-

quently hemolyzing the red cells from another person. When antibodies are present in the blood of one member of a species for the red cells of another member of the same species the antibodies are called *isohemolysins* and *isohemagglutinins*, respectively. It is quite important in transfusing a patient to select as a donor some one whose blood cells are not clumped by the serum of the recipient. Downs, Jones, and Koerber (1929) have shown that these isohemolysins require complement for their action as do other hemolysins produced by injection of foreign red cells. Isohemolysins and isohemagglutinins have been studied in rabbits, cattle and other animals. These substances and the inheritance factors involved will be discussed in another chapter under Blood Grouping.

ISOHEMOLYSINS IN PAROXYSMAL HEMOGLOBINURIA.—Another type of isohemolysin is described by Donath and Landsteiner (1904). This interesting type of hemolysin is apparently responsible for the clinical condition known as paroxysmal hemoglobinuria. They found in the serum or plasma of patients suffering from this disease, antibodies (isolsins) which would combine with the patient's own red cells *only* at low temperatures. They reasoned that the red cells of patients having these antibodies would become sensitized in the skin capillaries when the surface of the body was exposed to cold and would combine with complement after sensitization. When the sensitized red cells with their complement were transported to the internal organs where a temperature of 37° C. would be encountered, hemolysis would occur. Mackenzie (1927) has reviewed this subject and gives an excellent discussion of the work of Donath and Landsteiner and others.

Apparently the blood serum of almost every case of paroxysmal hemoglobinuria reacts with Wassermann antigens to give a positive serological reaction. While 24 to 25 per cent give a history of syphilis, it appears that approximately 75 per cent are negative as far as history goes; the diagnosis being made upon the positive serological findings. This is interesting in view of the work on natural antibody-like substances (reagins) that are found quite extensively in the blood of lower animals and to some extent in the blood of normal human individuals. Kahn (1940, 1941) has shown that these antibodies which unite consistently with Kahn antigen and less extensively with Wassermann antigen do so more

effectively at low temperatures, while syphilitic reagin or antibodies react more effectively at body temperature.

According to Mackenzie, Burmeister found that the lysins, dissociated from sensitized erythrocytes, give a positive Wassermann reaction. In his opinion there are some cases of paroxysmal hemoglobinuria which give a positive Wassermann test that are not syphilitic in nature while others show a relationship between the two diseases.

For more recent work upon the subject of natural reagins or antibodies having an affinity for antigens used in the diagnosis of syphilis, see the review of the literature by Kemp (1940) and experimental studies by Kahn (1940-1941), Sherwood, Bond and Clark (1941); and Sherwood, Bond, and Canuteson (1941).

The interesting point about these normal biological reagins reacting with Wassermann antigen is that they are another example of antibodies that will react at low temperatures much more intensely than they do at body temperatures. There is no reason to assume that they are the same antibodies that are present in paroxysmal hemoglobinuria because these normal reagins are present in the lower animals as well as in many cases of tuberculosis, leprosy, malaria, and in certain types of far advanced malignancies that do not show hemoglobinuria. It is possible that individuals with paroxysmal hemoglobinuria may have malaria, tuberculosis, and syphilis but the occurrence is probably a coincidence.

REAGINS.—ATOPIC REAGINS are antibody-like substances discovered by Prausnitz and Küstner (1921) in the blood of patients suffering from hay fever or asthma. Küstner was sensitive to fish. He found that when some of his serum was injected intradermally into the forearm of an individual not sensitive to fish the area of skin so infiltrated became temporarily sensitive to an extract of fish proteins. Reagins for the various pollens have been found in the blood of pollen hay fever and asthma cases. Coca suggested that these antibody-like substances be called "reagins" rather than antibodies. He took this position because, at that time, he did not believe that these substances developed as a result of antigenic stimulation in individuals possessing the necessary inheritance factor for allergy. This question will be discussed more fully in another chapter.

LIPIDOPHILIC REAGINS.—These are antibody-like substances that will sensitize emulsions of certain tissue lipoids in the same way that bacteria are sensitized by their specific antibody. Just as sensitized bacteria may become agglutinated or may unite with complement, so will sensitized lipid particles undergo agglutination if conditions are appropriate or they will combine with the complement if it is present. In man, reagins for acetone insoluble lipoids obtained from heart muscle or other animal tissue are found almost exclusively in the blood during the secondary and later stages of syphilis. A somewhat similar reagin, found occasionally in nonsyphilitics and quite extensively in the blood of the lower animals, can apparently be differentiated from syphilitic reagin by methods to be discussed in a later chapter.

PRECIPITINS.—These are antibodies produced against any unaltered soluble protein. They are usually found in the blood of any man or lower animal that has received injections of foreign protein as, e.g., antitoxin, normal horse serum, bacterial extracts, etc. The antibody sensitizes each colloidal particle of protein and this leads to a precipitation of these sensitized colloidal particles and to the union with complement if it is present.

ANAPHYLACTIC SENSITIZERS.—Many persons have considered these interesting antibodies as identical with the precipitins but more recently some doubt has been cast upon this hypothesis. They are antibodies that have the rather unique property of attaching themselves to smooth muscle and other tissue cells of the animal which produce them, several days after they make their appearance in the blood stream. This is rather a serious state of affairs for the animal, because on receiving another injection of antigen it would combine with these antibodies that are attached to the body cells and produce a serious physiological reaction called *anaphylactic shock*.

ANTITOXINS.—When a specific antigenic poison such as diphtheria toxin, tetanus toxin, botulinus toxin or streptococcus scarlet fever toxin is injected into an animal, a specific neutralizing antibody called *antitoxin* is developed for the particular kind of toxin injected. It neutralizes but does not destroy the toxin. Small amounts of antitoxins may be found in some normal animals and in man.

OPSONINS AND BACTERIOTROPINS represent normal and immune antibodies, respectively, that unite with specific bacteria and render them more easily engulfed by leucocytes. They are discussed more fully in another chapter.

ABLASTIN.—This relatively new antibody was discovered by Taliaferro (1929, 1932). It develops in rats infected with *Trypanosoma lewesi* and prevents reproduction or cell division of the parasites without apparent injury to them. It differs from other antibodies in that it does not form a demonstrable union with the cells (trypanosomes) which it specifically affects. This type of antibody is not produced as a result of infection with *T. brucei*, *T. gambiense*, *T. rhodesiense*, *T. equiperdum*, nor has Taliaferro observed it in his studies of the resistance of birds and mammals to certain malarial parasites.

ANTIAGGRESSINS.—Pathogenic bacteria vary in their ability to invade the tissues. Marked difference is exhibited by different strains of any one pathogen. Even some strains of *C. diphtheriae* seem to possess invasive powers. This is suggested by the work of Feierabend and Schubert (1929) and others to which attention has been called recently by Wells (1932). There are immunologists who interpret their experiments as showing that bacterial invasion is prevented by specific antibodies called by them *antiaggressins*. Their conclusions are based upon the acceptance of Bail's theory of aggressins. The veterinary immunologists have obtained startling results by immunizing domestic animals with antigenic material which they believe contains aggressins. Their work deserves serious consideration by the medical student since their experimental studies are both extensive and intensive. Many important papers have appeared in the *American Journal of Veterinary Medicine* and elsewhere in veterinary literature.

CONGLUTININS.—Ehrlich and Sachs (1902) discovered that inactivated bovine serum intensified hemolysis due to amboceptor and complement. Bordet and Gay (1906) and later Bordet and Streng (1909) investigated the phenomenon described by Ehrlich and Sachs and definitely showed that bovine serum contains a colloidal substance named by them *conglutinin* that unites only with sensitized cells that have adsorbed complement. It intensifies both the agglutination and hemolysis of such cells. They showed that this conglutinin will not unite with either unsensitized or

sensitized cells alone but will unite with the latter only after they have combined with complement. These authors also found that the substance resists heating to 56° C. and that it has biochemical properties somewhat similar to the albumins. Manwaring (1906) described a thermostable component of bovine serum which he named "auxilysin." It is probably the same as conglutinin.

HETEROPHILE ANTIBODIES AND HETEROPHILE ANTIGENS.—In 1911 Forssman discovered that a great variety of apparently otherwise unrelated substances possessed in common an antigenic factor that would stimulate rabbits to produce hemagglutinins and hemolysins for sheep cells. These nonspecies specific antigenic substances have been called heterophile antigens and the hemagglutinins and hemolysins resulting from their injection are called heterophile antibodies. The heterophile antigen present in sheep cells is distinct from the isophile or species specific antigen. This work was extended by Forssman and Hintze (1912), Forssman and Fox (1914), Pick (1913), Doerr and Pick (1913), and many others. More recent reviews and investigations are those of Taniguchi (1921), Landsteiner and Simmons (1923), Powell (1926), Hooker (1926), Bull (1928), Jungeblut (1929), Landsteiner and Levine (1932) and Meyer and Morgan (1935). As a result of this work the following conclusions relative to heterophile antigens have been formulated and generally accepted. They have been reported as present in the tissues (especially the kidney) of the guinea pig, horse, cat, dog, camel, mouse, chicken, gills of carp and pike, in the plasmas and urine of the horse, in the red blood cells of the sheep, goat and chicken and also in certain bacteria such as *B. enteritidis* of Gaertner, *B. paratyphosus* B and *B. dysenteriae* (Shiga). As a rule, with the exception of the chicken, when the heterophile antigen occurs in the tissues of an animal, it is absent from the red cells of that animal and conversely, when it is present in the red cells, it is absent from the tissues. It is interesting to note that while it is found in the chicken and in the mouse, it is not present in either the pigeon or the rat. While it is present in *B. paratyphosus* B, it is absent from *E. typhosa*. The Forssman antigen found in many animal tissues seems to be made up of a protein and perhaps a combination of lipoid and polysaccharide whereas the active substance in bacteria seems to be polysaccharides alone. Schiff and Adelsberger (1924) and others seem to have established

a relationship between the group A substance of human red cells and the polysaccharide of Forssman's antigen. Interest in heterophile antibodies has increased since Paul and Bunnell (1932) noted their presence in high titer in many cases of acute infectious mononucleosis. The subject has been extensively investigated by Davidsohn.

While the term heterophile antibodies has been used almost exclusively to indicate antibodies for nonspecies specific antigens of the Forssman type which will cause agglutination of old sheep red cells, it should be remembered that there are other examples of nonspecies specific antibodies. Among the well-known antibodies that cause cross reactions are those that are produced by immunizing with Friedländer's bacillus. Such antibodies will agglutinate not only suspensions of *B. fridländer* but also suspensions of *Pneumococcus* Type II. Another example of heterophile antibodies are those found in the blood of typhus fever patients. These antibodies will react with both *Proteus* OX19 and the *Rickettsia* of Mexican and European typhus fever. While these antibodies giving cross reactions are for nonspecies antigens and therefore are heterophile antibodies they do not cause agglutination or hemolysis of sheep cells.

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CHAPTER IX

COMPLEMENT

Complement.—**DEFINITION.**—Complement may be defined as a thermolabile enzyme-like colloid, present in the serum of animals, that has the ability to combine with sensitized cells and bring about their lysis under suitable conditions.

The history of the discovery and naming of this substance is discussed briefly in an earlier chapter. It was regarded as an enzyme by Buchner who named it alexin and regarded it as of leucocytic origin. Ehrlich regarded it as having a combining or haptophore group and a zymophore or enzyme-like group which was responsible for lysis. Some of its enzyme-like properties may be summarized as follows: (1) It is colloidal in nature. (2) The presence of a small amount brings about extensive chemical changes in the substrate (cells). (3) It is thermolabile. (4) The reaction is about as reversible as enzyme reactions. (5) The optimum temperature for its action is 37°C . (6) Antienzymes as well as anticomplement have been reported. (7) There is considerable evidence that its activity is interfered with by the end products of its action.

NATURE OF COMPLEMENT.—Biochemically speaking, Ferrata (1907) reported that complement is composed of an albumin fraction, named by Brand (1907) "endpiece," and a globulin fraction which he calls "midpiece." Subsequent work by Whitehead, Gordon and Wornall (1925) indicates that the midpiece consists of a heat labile euglobulin fraction and a heat stable fraction named the third component. The latter can be removed from a serum by treatment with zymim (residue after acetone and ether extraction of yeast) and restored by adding guinea pig serum heated to 56°C .

In 1926 the same authors reported that complement could be rendered inactive by treatment with appropriate concentrations of ammonia and that the activity could be restored by the addition of either heated or zymim-treated serum. They named this ammonia sensitive fraction the "fourth" component and determined that it is associated with the endpiece. Pillemer, Seifter

and Ecker (1941) have made an extensive study of the endpiece including the fourth component of complement. They say that their findings "seem to indicate that the endpiece of complement is the calcium-carbohydrate-pseudoglobulin molecule" and that in the light of this "the fourth component would be the carbohydrate." They consider that the complex calcium-pseudoglobulin molecule is the carrier of the active carbohydrate factor. If their work is confirmed, the old conception of endpiece as an albumin will have to be abandoned.

In another paper Ecker, Jones and Kuehn (1941) suggest that the euglobulin fraction of midpiece is closely associated with a phosphatide (cephalin). The importance of this observation is apparently unknown. Sensitized cells which have adsorbed the globulin fraction (midpiece) only are called "per sensitized" cells.

PRESERVATION OF COMPLEMENT.—When serum rich in complement is frozen, its potency as a lytic agent of sensitized cells is retained for one or more weeks. Kolmer suggests that this is probably the oldest method of preserving complement. Morgenroth and others were able to preserve complement kept at -10 to -15° C. for several weeks. Kolmer found that either diluted or undiluted complement kept frozen except when samples are removed retains its titer undiminished for 3 or 4 days, after which time it drops rather rapidly. The titer of undiluted complement kept at 4° to 9° C. is diminished in 24 hours, and shows progressive deterioration thereafter.

Many laboratories preserve complement by freezing and desiccation in vacuo at a low temperature. This method has come into rather general use since the publication of Flosdorf and Mudd (1935, 1938) in which they describe the procedure and apparatus that operates successfully. The methods are applicable to the preservation of various kinds of immune sera and also bacteria. The first method depended largely upon the vacuum pump for removal of the water vapor given off during drying and the dried serum was called "lyophile" serum. In 1938 the same authors reported upon a simpler and better method of desiccation. In this method both chemical desiccants and physical evacuation were used. The method is called the Cryo-chem process. Complement preserved by this method is stored in the refrigerator and maintains its titer for long periods of time.

A method which has been used with success in this laboratory is given by Boerner and Lukens (1939). This is a modification of the original procedure of Sonnenschein. The serum is obtained and an equal volume of the following mixture is added: sodium acetate—12.0 grams; boric acid—4.0 grams; sterile distilled water q.s.—100.0 c.c. The mixture is then stored in the ice box and will keep for relatively long periods of time.

EFFECT OF HEAT ON COMPLEMENT.—The optimum temperature for complement action is 37°C ., but it loses its activity within a few hours at this temperature. Manwaring (1906) studied the effect of higher temperatures on complement and found that noticeable diminution in titer occurs within 10 minutes at 49°C ., although traces are present at the end of one hour. At 51°C ., there is greater diminution in 10 minutes and complete inactivation in 35 minutes. When a temperature of 53°C . is employed for 14 minutes the lytic property of complement disappears completely. He found also that complete inactivation occurs in 12 minutes at 55°C ., 8 minutes at 57°C ., 4 minutes at 59°C ., and 2 minutes at 61°C .

REVERSIBILITY OF THE REACTION.—Gramenitski (1912) suggests that the inactivation of complement at 56°C . is not an irreversible reaction. Brooks (1919, 1921) has confirmed these observations and concludes that under proper conditions a partial return of activity may occur. Just how inactivation is brought about by heat is not definitely known. It is thought that a change in colloidal dispersion is an important factor.

INACTIVATION BY SHAKING.—This concept is supported by the observations of Jacoby and Schütze (1910) that prolonged shaking will eventually lead to complement inactivation. This has been confirmed by a number of workers.

ANTICOMPLEMENTARY EFFECTS OF INORGANIC AND ORGANIC COMPOUNDS.—Hektoen (1903), Manwaring (1904) and others have shown that definite concentrations of various inorganic salts inhibit complement action. In 1913, Arkin reported that a number of inorganic substances, as well as lactic acid, interfere with phagocytosis and concluded that it is due to an effect upon complement. A few years later Sherwood (1917) found that many other inorganic and organic compounds will prevent the lytic action of complement when present in very small concentrations.

Mention has already been made of the effect of zymine and ammonia upon midpiece and endpiece, respectively. These results indicate the necessity of using chemically clean glassware in all immunological work dealing with opsonins and complement fixation.

ORIGIN OF COMPLEMENT.—In regard to the origin of complement nothing is known definitely, nor is it absolutely settled that it exists as such in the circulating plasma. Buchner (1892), Hankins (1892) and Metchnikoff suggested, quite early, that it was of leucocyte origin. In connection with this, it is of interest to note that when sensitized red cells are phagocytized by leucocytes, they seem to be no more readily hemolyzed than unsensitized cells. A great many have reported that the complement titer of serum is higher if it is allowed to stand on the clot in the refrigerator at 6 to 8° C., for 24 hours. It has been argued that this increase in titer is due to the leucocytes present. Regardless of theoretical considerations, the observation that an increase in titer frequently occurs under the conditions just mentioned is of practical importance in diagnostic serology. A great many have offered experimental evidence that the liver is the source of complement. Sherwood, Smith and West (1916) investigated this question and concluded that it is neither the only, nor the principal source of complement. They point out important sources of error in the work of Dick and of Nolf and others who had concluded that complement is of hepatic origin.

DESIRABLE QUALITIES OF A COMPLEMENT.—For use in complement fixation, there are certain qualities that must be considered in choosing complement. It should be readily fixed by a sensitized antigen; free from natural hemolysins, and agglutinins; lysis should be reasonably rapid, and it should be sufficiently stable for use in overnight fixation at 6° to 8° C.

COMPLEMENT FROM VARIOUS ANIMALS.—Guinea pig complement most nearly meets these requirements, although human complement is quite satisfactory. The complement titer varies from day to day in the same pig, and may be nil in apparently healthy guinea pigs. For these reasons it is recommended that pooled complement (from 3 or 4 guinea pigs) be used in complement-fixation tests. A satisfactory unit of guinea pig complement in the Kolmer-Wassermann technique should be from 0.25 to 0.35 c.e. of a 1:30

dilution, and hemolysis should be practically complete after twenty to thirty minutes in a water-bath held at 37° C., although the period of incubation is one hour. Rabbit complement and dog complement are very unsatisfactory, since nonspecific fixation is quite commonly encountered.

Bond and Sherwood (1939) found that the hemolytic property of snake serum is due to the action of a thermolabile complement and a naturally occurring hemolysin. The complement in snake serum possesses properties similar to guinea pig complement. The titer is usually high; it is thermolabile and deteriorates on standing. Furthermore it was bound by a specific bacterial antigen-antibody complex and also by the syphilitic antigen-reagin complex respectively.

SPECIFIC FIXATION OF COMPLEMENT.—In 1901 Bordet and Gengou observed that sensitized bacterial cells adsorbed or bound complement and apparently proved that the complement that lysed sensitized bacteria was identical with the complement that laked sensitized red cells. This work, however, has not ended the controversy over whether there is one complement or many complements, but it did give the scientific world the complement fixation technique. The subject of multiplicity or singleness of complement is discussed rather extensively by Zinsser, Enders and Fothergill (1939).

SPECIFIC COMPLEMENT FIXATION BY PRECIPITATES.—The following year (1902) Gengou showed that precipitates formed in the precipitin reaction would bind complement. Gengou explained this as due to amboceptors or sensitizing antibodies present in the immune serum along with precipitins. According to the unitarian viewpoint supported by Zinsser and others, the sensitizer and precipitin are identical, and while flocculation does not require complement, the precipitate formed, which is composed of sensitizer and antigen, is able to adsorb complement. Experimental support for this has been offered by Gay (1905), Moreschi (1905), and others. It was Gay who noted the relationship to the precipitin reaction.

TIME OF OCCURRENCE OF FIXATION.—Dean (1913) has shown that the rate and character of precipitates formed influence the fixation of complement. In his opinion the greatest fixation of

complement occurs early in the reaction before large aggregates are formed and when the optimum surface of sensitized colloid is available for absorption.

NONSPECIFIC ADSORPTION.—Cells whose surface contains lipoids seem to be able to adsorb complement in a nonspecific manner. Landsteiner and von Eisler suggest the importance of lipoids in certain nonspecific complement fixations. They conclude, however, that the colloidal state is the important factor in this nonspecific fixation. Wilde (1901) noted that emulsions of most bacteria adsorb complement even when not sensitized. This is important in view of the use of bacterial complement fixation in the diagnosis of certain diseases as well as in the identification of micro-organisms.

CHEMICAL AND PHYSICAL FACTORS IN CELL SENSITIZATION.—In other chapters cell sensitization, or the union of antigen and antibody, is discussed extensively. The phenomenon of specificity which seems to depend upon the chemical constitution of the antigen forces one to recognize definite chemical factors in the process. The evidence seems to bear out Bordet's contention that adsorption plays a prominent part and that the antibody globulin appears to become insoluble in saline. The sensitized cells behave in cataphoretic experiments like particles of denatured globulin. The reaction occurs quite readily at 0° C. Purely chemical reactions occur very slowly in aqueous systems at low temperatures, but the rate is greatly accelerated as the temperature is increased. Adsorption phenomena, on the other hand, are noticeably increased with the lowering of temperatures, and hence occur readily at 0° C.

At the present time there are two concepts of adsorption that should be borne in mind. Adsorption may be illustrated by the removal of coloring matter from water by means of powdered charcoal. According to one theory, this is accomplished by the molecular capillary state of the surface, while according to another concept the valences of the molecules within the charcoal are all satisfied because they are united with each other, but those at the surface have unsatisfied valences or fields which unite with the coloring structure and physical state of the dye as well as the adsorbing surface. It would seem probable that either or both

mechanisms might be included under the term adsorption, since they would represent methods of surface binding of matter.

THIELE AND EMBLETON'S OBSERVATIONS.—Another interesting observation concerning the union of antibody with antigen is reported in an excellent paper by Thiele and Embleton (1914). They showed that a given amount of washed red blood cells is sensitized by definite amounts of antibody regardless of the concentration of antibody per cubic centimeter. In other words, if a sensitizing dose of amboceptor is added to one cubic centimeter of salt solution or 20 c.c., it is removed by adding the same unit of red cells to each tube.

BORDET'S AND EHRLICH'S VIEWS ON THE MECHANISM OF SENSITIZATION.—As previously stated, Bordet showed that this process of sensitization of antigen occurs before complement could bring about lysis, since the latter acted only upon sensitized cells. Bordet concludes from his experiments that complement is adsorbed by the antigen-antibody complex with resulting lysis, while Ehrlich concludes that amboceptor chemically combines with antigen on the one hand and with a combining group of complement on the other. The complement is thus enabled to act on the antigen, producing lysis.

VISIBLE PHENOMENA OF CELL LYSIS.—At this point it might be well to visualize what can be seen when an adequate amount of complement is added to various sensitized antigens such as *Microspira comma*, *E. typhosa*, immune precipitates and sensitized red cells.

In the case of a sensitized suspension of living *Microspira comma* there occurs at 37° C. at first a rounding up of the organisms, followed by their disappearance. They seem to be almost completely destroyed except for a residual granule. Living sensitized typhosus suspensions are killed, as can be demonstrated by plating methods, but the individual cells frequently appear to be unchanged. Sensitized streptococci, staphylococci and also sensitized precipitates seem to show no change. When sensitized red cells are mixed with an adequate amount of complement, there occurs after a few minutes a swelling of the cell and liberation of the hemoglobin; the stroma or cell framework remains.

FIXATION OF COMPLEMENT BY PRODUCTS OF LYSIS.—Liefmann and Cohn (1910) showed that during hemolysis there are liberated products which render complement inactive. A number of workers have considered that complement functions as an enzyme and is bound by the products of lysis rather than by the antigen-antibody complex.

HILL AND PARKER'S PHYSICOCHEMICAL INTERPRETATION.—Hill, Parker and McKinstry (1925) give a physicochemical interpretation to a simple equation which they believe explains all the phenomena of hemolysis by complement. In their paper they draw the following interesting conclusions: That complement is a catalyst. It enters into and combines with the cell under the influence of amboceptor. That the latter (amboceptor) contains a ferment which can bring about the release of hemoglobin. Complement acts as a catalyst for this reaction. They conclude that complement is fixed by some other fraction of the firmly bound amboceptor and that the quantity of this complement fixing substance does not change during hemolysis. This hypothesis is interesting since it is based upon an equation which fits not only their own experimental results but also those of Thiele and Embleton, and certain concepts advanced by Nolf in 1900. Undoubtedly there are many secondary factors introduced when the antigen undergoes visible physical and chemical change such as occurs with *Microspira comma* or with red cells, which are not present in reactions of complement with other sensitized bacteria or sensitized inert particles.

ACTION OF COMPLEMENT DEPENDS ON CONCENTRATION.—While it is obviously difficult to be certain of the exact mechanisms involved, there is no difference of opinion over certain observed facts about complement. Thiele and Embleton find that whereas amboceptor unites with antigen regardless of the amboceptor concentration per cubic centimeter, complement action depends upon the existence of a certain minimum concentration of complement per cubic centimeter of the solution. In other words, there might be enough complement in a test tube to hemolyze 50 or more units of red cells, but unless its concentration per cubic centimeter is adequate no hemolysis will result. Others have shown that while antigen and antibody unite readily at 0° C., complement and sensitized antigen do not, although they do unite at temperatures slightly above zero.

This union at low temperatures makes possible a primary incubation of 6° to 8° C. in complement fixation work.

EFFECT OF TEMPERATURE ON COMPLEMENT ACTION.—In regard to the effect of temperature upon complement action, Zinsser says the speed and completeness of the union of complement and sensitized antigen increase as the temperature approaches 40° C.

DISCUSSION OF EAGLE'S WORK.—Eagle (1929) has reported a series of experiments attempting to explain the mechanism of complement fixation. He finds, as others before him, that qualitatively speaking, the phenomenon of hemolysis appears to represent a monomolecular reaction, and that shortly after hemolysis appears, there is an increase in the rate of fixation of complement. He studied and compared fixation of complement by agglutinated bacteria, immune precipitates and sensitized red cells, and noted a similarity in the curves, with the exception of this added fixation in the case of hemolysis. Liefmann and Cohn (1911) noted this, as did Thiele and Embleton (1914). While it is an interesting observation, it does not prove the monomolecular nature of the reaction, nor is it so regarded by Eagle. He concludes that "the physical constants of fixation (temperature coefficient, velocity, quantitative relationships between the reactants) are those commonly associated with adsorption processes and are the same in the three types of fixation studies."

While this is a good working explanation of complement fixation, it should be remembered that his conclusions result largely because his data fit Freundlich's equation for adsorption processes and also because the figures he obtains for temperature coefficients are low as is the case in adsorption reactions. The Freundlich equation is usually used in the sense of circumstantial evidence rather than proof, and the problem is such that it is doubtful whether the temperature coefficients he reports are significant. Since this explanation of complement action is perhaps the most commonly accepted one, it can be used as a working hypothesis for the present. This admits that both chemical and physical factors are involved, and assumes that complement is bound to the antigen-antibody complex preliminary to any lytic action that may occur, and also admits of the interference of lysis by secondary products liberated or formed in the process.

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CHAPTER X

ISOHEMAGGLUTININS—BLOOD GROUPS

Isohemagglutinins.—**DISCOVERY.**—The first recorded observation that the serum of a normal, healthy individual can bring about agglutination of the red cells of other normal individuals of the same species was made by Landsteiner in 1900. This preliminary report was followed by an intensive study of the sera and red cells of twenty-two individuals. From an analysis of the data obtained in this study, Landsteiner (1901) discovered that three definite blood groups were represented. The following year DeCastello and Sturli (1902) continued this work at the suggestion of Landsteiner. They not only confirmed the existence of the three groups, but found four exceptions which are the first recorded examples of the fourth group. They even discussed four possible groups which they designated by numbers rather than by letters. Since the four exceptions that constituted a new blood group were bloods from young children, DeCastello and Sturli thought that they might be members of one of Landsteiner's groups that had not yet acquired their full quota of necessary factors. According to Ottenberg (1928) and also Zinsser and Coea (1931), Landsteiner immediately appreciated the existence of the four groups. He had previously postulated (1901) two agglutinins and two agglutinogens as was later done by Jansky (1907). Hektoen (1907) and Gay (1907) both confirmed the existence of the three human blood groups described by Landsteiner. Two years later Landsteiner (1909) published a more complete discussion of his work on isohemagglutinins. In 1910, Moss offered a classification of the blood groups which has found wide acceptance. Apparently Hektoen (1907), Gay (1907) and Moss (1910) were not aware that previous workers (Landsteiner, et al.) had considered more than three groups and had postulated two agglutinins and two agglutinogens since both Hektoen and Moss speak only of Landsteiner's three groups and certain exceptions and both assume the existence of three agglutinins. Landsteiner (1928), Ottenberg (1928) and Snyder (1929) have reviewed the literature

on isohemagglutination and have discussed various classifications and theories relative to the inheritance factors involved. Kennedy (1931) published an extensive review of Jansky's work. This is of interest since the original publication is found in few libraries.

CLASSIFICATION IN USE.—At the present time there are three classifications of the blood groups in use. In the classifications of Jansky and Moss the groups are numbered. While groups II and III are the same in both classifications, it should be remembered that group I of Moss corresponds to group IV of Jansky, and conversely, group IV of Moss is the same as group I of Jansky.

CLASSIFICATION BASED ON AGGLUTINOGEN CONTENT.—In the third and more recent classification, the groups are identified by the agglutinin content of the red cells. The cells of the first or "O" group are not agglutinated by the sera of any group and hence may be regarded as not containing an agglutinable substance or agglutinin. One can imagine that the letter "O" stands for the German word *ohne* meaning without. The red cells of the second or A group contain agglutinin A; of the third, agglutinin B, while cells of the fourth group contain both A and B agglutinin and hence the group is called AB. The order of arrangement corresponds to that of the Jansky classification. A comparison of these three classifications with each other and with Landsteiner's original groups is summarized in Table I:

TABLE I
COMPARISON OF CLASSIFICATION OF BLOOD GROUPS

Jansky	I	II	III	IV
Moss	IV	II	III	I
New Classification	O	A	B	AB
Landsteiner's original three groups	C	A	B	Type described by DeCastello and Sturli

LANDSTEINER'S THREE GROUPS.—In Table I it will be noted that Landsteiner used the letters A, B, and C to designate his three groups. He found that serum of group C agglutinated the red cells of groups A and B; the serum of group A agglutinated the cells of B but not those of C or A; and the serum of group B agglutinated the cells of A but not those of C or its own group B. On the other hand, the serum of the new type, or exception,

described by DeCastello and Sturli (1902) failed to agglutinate the cells of any group while the corresponding red cells were agglutinated by the sera of each of the three Landsteiner groups.

MECHANISM POSTULATED BY LANDSTEINER.—To explain these phenomena, Landsteiner (1901, 1928) says that he and later Jansky (1907) postulated the existence of two agglutinins, alpha and beta, and two corresponding agglutinogens, A and B. He furthermore assumed that there exists a reciprocal relationship between them; i.e., when an agglutinin is absent from the red cells of an individual the corresponding agglutinin will be present in the serum, and, conversely, when an agglutinin is present in the red cells, the corresponding agglutinin will be absent from the serum. This explains why the red cells are not agglutinated by the homologous serum.

Thus, for group O where the red cells contain neither agglutinin, the corresponding serum contains both agglutinins alpha and beta. In group A, the red cells contain agglutinin A, and hence group A sera will contain only beta agglutinin. Likewise, group B sera will contain only alpha agglutinin and group AB neither agglutinin.

This will be better appreciated from an inspection of Table II.

TABLE II

Agglutinin content of cells	O	A	B	AB
Agglutinin content of corresponding group serum	α, β	β	α	—
Jansky types	I	II	III	IV
Moss types	IV	II	III	I

Fig. 6 illustrates the phenomena observed from mixing the cells and sera of the four types.

MOSS'S CLASSIFICATION IN GENERAL USE.—Kennedy (1929) states that the classification suggested by Moss (1910) is used in 78 per cent of the hospitals of America. This is interesting in view of the fact that in 1921 a committee representing the American Association of Immunologists, the Association of Pathologists and Bacteriologists, and the Society of American Bacteriologists recommended the adoption of the Jansky classification. This was done

in recognition of the priority of Jansky's work. Since the majority of the hospitals insist upon using the classification of Moss it is necessary that this classification be taught to medical students.

Suggested Method for Learning the Classification.—Experience in teaching has shown that if the student will associate the new classification "O,A,B,AB," which is based upon the agglutinogen content of the red cells, with some sentence such as "Oh, \bar{A} , Be, \bar{A} -be," keeping in mind the law of reciprocal relation-

			Cells (agglutinogens)			
Moss	Jansky	Agglu- tinin Content	Moss IV O (Jansky-I)	Moss II A-(Jansky-II)	Moss III B-(Jansky III)	Moss I A-B (Jansky IV)
IV	I	$\alpha\beta$				
II	II	β				
III	III	α				
I	IV	-				

Fig. 6.—Distribution of agglutinins and agglutinogens in human blood groups.

ships between agglutinin and agglutinin postulated by Landsteiner, he has no difficulty in remembering and understanding this classification. It is also our experience that he has no difficulty in remembering that the numerical groups of Jansky correspond to the "O,A,B, and AB" groups of the new classification and that in Moss's classification groups I and IV of Jansky's are reversed. By learning the new classification and the law of reciprocal relationships he has learned the agglutinin and ag-

glutinin content of each group of both the new classification and that of Jansky and hence can readily determine the same for the groups of Moss.

TIME OF APPEARANCE OF AGGLUTINOGEN AND AGGLUTININS.—Since the discovery of the blood groups of Landsteiner, numerous investigations have uncovered many interesting facts relative to the factors involved in isohemagglutination. It has been shown, for instance, that the agglutinin content of the red cells is, as a rule, complete at birth. On the other hand, the agglutinins may or may not be present in the serum at birth, but may make their appearance at some time during the first four years of life. After the isohemagglutinating factors of a blood are complete, there is no qualitative change during the lifetime of the individual. It has been shown that disease may increase or decrease the titer and that the titer may drop during the later years of life, but the group, when once determined, remains constant. A number of individuals have found agglutinins from the maternal blood present in the blood of the child at birth. These usually disappear from the blood stream by the tenth day. Various theories have been offered to explain their presence in the circulation of the newborn. It is thought that changes in permeability of vessels in the placenta may account for the phenomenon.

Inheritance of Blood Group Factors.—The first evidence suggesting that the blood group factors might be inherited was presented by Epstein and Ottenberg (1908). They typed a mother and seven sons of one family and four sons and both parents of another family. All members of the first family were found to be in group A, while all members of the second family were in group B. Two years later, von Dungern and Hirschfeld (1910) obtained data on seventy-two families covering two generations and 248 individuals. From an analysis of this data, according to Ottenberg* (1928) and Snyder (1929), they concluded: “(1) A or B never occurs in the red cells of a child if not present in one of the parents. (2) When one of these substances is present in both parents it occurs in most of the children. (3) When only one parent has one of these particular substances, some of the

*Ottenberg and Beres: *Newer Knowledge of Bacteriology and Immunology*, Jordan and Falk, Univ. of Chicago, 1928, p. 909.

children inherit it. (4) When a particular substance is absent from both parents, no child ever has it."

RACIAL DISTRIBUTION OF GROUPS.—In 1919 L. and H. Hirschfeld published the results of their studies of the distribution of human blood groups among sixteen nationalities including over eight thousand individuals. The data were obtained during the World War while they were serving as army physicians on the Balkan Front. They observed a significant difference in frequency of occurrence of the agglutinogens among different races. Since then, many additional studies have been made.

RACIAL TYPES.—The data obtained by the Hirschfelds indicate that the nationalities may be grouped into three types (see Snyder, 1929, p. 118) the European, Intermediate and Asio-African types. In the European type, which is made up of the English, French, Italians, Germans, Austrians, Serbians, Greeks and Bulgarians, the frequency of the A factor is quite constant, varying from 41.8 in the Italians to 48 in the Germans. On the other hand, the B factor shows a progressive increase from 10.2 for the English to 20.4 for the Bulgarians. In the Intermediate group, composed of Arabs, Turks, Russians and Jews, the frequency of the A factor still exceeds that for B, but they are noticeably closer together. The Asio-African type is composed of the Madagascans, Senegalese, Annamese and natives of India. In this group the frequency of the B factor is equal to or definitely greater than that for the A factor. In these determinations, the data for the A factor include A and AB, while those for the B factor include both B and AB. Ottenberg (1928) has also given an excellent summary of the frequency of blood groups in different populations.*

FREQUENCY IN DIFFERENT POPULATIONS.—Apparently the frequency of the O group varies between wide limits. The Chinese show a frequency of 30; the Russians, 40.2; Germans, 40; French, 43.2; English, 46.4; Australian aborigines, 51; and the American Indians, 77.7 to 91.3 according to different investigators.

HIGH INCIDENCE OF O GROUP IN THE AMERICAN INDIAN.—The American Indians have been studied by Coca and Deibert (1923), Snyder (1926) and Nigg (1926). It is interesting to note that all three studies have shown a high incidence for group O and a

*It is interesting to note reported variation in the incidence of the N factor. Vibeke Fabricius-Hansen finds it lower in the Greenland Eskimos and American Indians than in other peoples, *J. Immunol.* 38: 405, 1940.

very low incidence for B and AB. The frequency of B varied from 0.22 to 2.1 while AB varied from 0.0 to 0.22. Nigg (1926) has also published data from this laboratory covering her investigations on the incidence of the various groups in 413 full-blooded Hawaiians. She found 36.5 per cent were of group O, 60.8 per cent in group A, 2.2 per cent in group B and 0.5 per cent in group AB. Nigg (1929), as well as Coca and Deibert (1923), concludes that the highly civilized races are characterized by the presence of isoagglutinogens, whereas the primitive peoples in all parts of the world are characterized by the low incidence of isoagglutinogens. This conclusion would seem to be borne out by the work of Goodner (1930) who found that of 223 pure Maya Indians studied, 97.7 per cent belonged to group O. Rife (1932) likewise studied the incidence of blood groups among the Indians in certain Maya areas of Central America. He found that 122 out of 124 belonged to group O. Recently Matson and Schrader (1933) reported that the Blackfeet and Blood tribes of American Indians show a high percentage of group A and a relatively low percentage of group O. They found 76.5 per cent of 115 allegedly full-blooded Blackfeet and 20 out of 24 full bloods of the Blood tribe belonged to group A. Perhaps later work will explain these exceptions.

Distribution of Agglutininogen in Lower Animals.—The distribution, among the lower animals, of substances either similar to or identical with the agglutinogens found in human red cells has been studied extensively by Landsteiner (1902), von Dungern and

TABLE III
AGGLUTINATION TESTS ON ERYTHROCYTES OF OTHER ANIMALS

	MOUSE	RABBIT	RABBIT	RAT	GUINEA PIG	DUCK	PIGEON	GOOSE	DONKEY	HORSE	PONY	GOAT	CAT	DOG	HUMAN II	HUMAN III
Agglutinin solution from human group II serum	+	++	++	++	+	0	0	0	+	0	0	0	+	++	0	+
Agglutinin solution from human group III serum	0	Tr?	+	Tr.	0	0	0	0	0	0	0	0	0	Tr?	+	0

0 Negative. Tr. Trace of agglutination. Degree of agglutination indicated by ±, +, ++, etc.

From Landsteiner and Miller: J. Exper. Med. 42: 869, 1925.

Hirschfeld (1910-11), Hooker and Anderson (1921), Landsteiner and Miller (1925) and also Landsteiner and Levine (1929, A and B).

Table III taken from the paper by Landsteiner and Miller (1925) shows an interesting distribution of agglutinogens in the blood of the lower animals. While many of these are similar to certain agglutinogens in human blood they are not identical.

For further information concerning the occurrence of receptors similar but not identical with human substances A and B in the

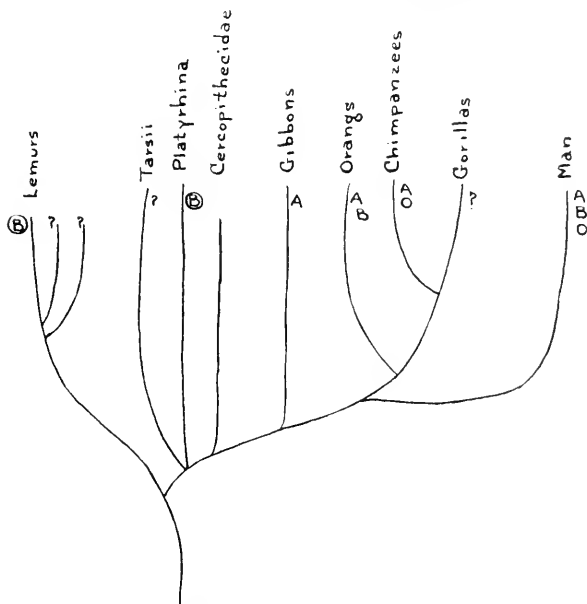


Fig. 7.—Graph showing distribution of agglutinogens in apes resembling those in man. Suggested by illustration in paper by Landsteiner, K., and Miller, C. P., Jr., *J. Exp. Med.* 42: 871, 1925.

blood of the lower animals the student should consult the reviews of Wiener, Landsteiner and Snyder.* Bond† (1939) has shown that snake blood contains agglutinins for the A and B factors of human cells but that no A and B factors were present in the snake cells.

AGGLUTINOGEN IN MONKEYS AND HIGHER APES.—In their studies on the bloods of the lower monkeys and also of the anthropoid apes, Landsteiner and Miller found an agglutinogen similar but not identical to the human B factor, in the red cells of Lemurs, Platyrrhinae, and Cercopithecidae. The red cells of the Gibbons

*Ferguson identified nine antigens in the erythrocytes of cattle, *J. Immunol.* 40: 213, 1941.

†Bond has also found similar agglutinins and also a species specific one in turtles but not in alligators, *J. Immunol.* 39: 125, 133, 1940.

contained A, the Orangs, both A and B and the Chimpanzees, the A agglutinin. The serum of the latter also contained agglutinins corresponding to those of the human O group.

The biological relationships of the monkeys and apes, correlated with the various agglutinogens, are quite well illustrated in Fig. 7 suggested by the illustration used by Landsteiner and Miller (1925).

Comparison of Agglutinin in Human and Monkey Blood.—

In regard to the agglutinin found in the red cells of Lemurs and Platyrrhinae that is similar to but not identical with the B factor of human cells, Landsteiner and Miller state that the difference is detected when these monkey blood cells are used to absorb the beta agglutinins from type II human serum. They are unable to remove all of the agglutinin although unabsorbed type II serum agglutinates the cells and type III serum does not, or, at most, gives only slight agglutination.

On the other hand, Landsteiner and Miller* (1925) have shown that the bloods of anthropoid apes "contain groups apparently identical with those of human blood." They were able to assign each blood to one of the four human groups. In regard to the bearing of these results upon the question of the origin of the blood groups in Man, they make the following statement: "If our findings in the anthropoids are taken into consideration, the simplest assumption seems to be that the isoagglutinable factors existed before Man and the anthropoids were differentiated from their common ancestor. If this assumption is not made, one is forced to the conclusion that identical mutations occurred in the evolutionary lines which developed into the Gibbon, Orang, Chimpanzee and Man at some later time."

HUMAN A AND B FACTORS IN ANTHROPOID APES.—They also call attention to the interesting distribution of the agglutinogens in the anthropoid apes. Apparently, the Chimpanzee has only the A factor and its blood grouping A and O is comparable to that of the American Indian, while the Orangs contain both the A and B factors similar to those of human races other than the Indians.

Medico-Legal Application of Blood Groups.—A great deal of attention has been given to the mechanism of inheritance of the agglutinogens A and B. Von Dungern and Hirschfeld regarded

*Landsteiner and Miller: J. Exper. Med. 42: 841, 1925.

the two agglutinogens and their corresponding agglutinins as similar to independent factors described by Mendel in his experiments with peas. They considered that the agglutinogen factors A and B are dominant to the corresponding recessives a and b. They represented group O genetically, which is devoid of agglutinogens, by the double recessive aabb (see Ottenberg, 1928, 912). Table IV, taken from a paper by Ottenberg (1928), shows the genetic formulas of the four groups as suggested by von Dungern and Hirschfeld:

TABLE IV
GENETIC FORMULAS OF THE FOUR GROUPS*

O	A	B	AB
aabb	AAbb Aabb	aaBB aaBb	AABB AaBB AABb AaBb

Table IV shows all of the possibilities according to the two-factor hypothesis of von Dungern and Hirschfeld. For a more comprehensive discussion of the genetic factors involved, the student is referred to an excellent book by Snyder (1929).

Ottenberg (1928) has also included Table V showing the groups of the children resulting from all the possible crosses under this two-factor theory.

TABLE V*

PARENTS	CHILDREN POSSIBLE	CHILDREN NOT POSSIBLE
O x O	O	A, B, AB
O x A	O, A	B, AB
O x B	O, B	A, AB
A x A	O, A	B, AB
A x B	O, A, B, AB	
A x AB	O, A, B, AB	
B x B	O, B	A, AB
B x AB	O, A, B, AB	
AB x AB	O, A, B, AB	
O x AB	O, A, B, AB	

DETERMINATION OF NONPATERNITY.—Ottenberg* calls attention to the possibility of “ruling out the reputed father as definitely not the father of the child being examined” by a reference to the

*From “The Heredity of the Blood Groups” by Ottenberg, R., and Beres, D., in *The Newer Knowledge of Bacteriology and Immunology*, edited by E. O. Jordan and I. S. Falk. Reprinted by permission of the University of Chicago Press.

above table after the groups of the parents and child are determined. It will also be noted that there are limitations to one's ability to determine paternity by means of isoagglutination tests. For a more comprehensive discussion of this subject the student is referred to the studies of Ottenberg (1928), Snyder (1929), Hooker and Boyd (1929), Wiener, Lederer and Polayes (1930), and Wiener (1933).

Bernstein's Triple Allelomorph Theory.—For all practical purposes the assumption of Landsteiner that there are two agglutinogens and two agglutinins for which reciprocal relationships exist is quite satisfactory. However, the genetic formulas developed from such an assumption do not give data that explain the racial distribution of blood groups; Bernstein (1925) developed an hypothesis which enables him to write formulas that give data comparable to those observed. His theory is known as the three factor or triple allelomorph hypothesis. In this he assumes that the red cells of the O group contain a recessive agglutinogen designated by Snyder (1929) as O and that there exists a corresponding agglutinin \ddot{o} . He furthermore assumes that the O agglutinogen is present in some bloods of group A and also of group B but never in group AB. He postulates that all three agglutinins are present in all sera, but that there exists a reciprocal binding between an agglutinin and its corresponding agglutinogen that prevents autoagglutination. Thus he assumes the existence of six genetic types. Snyder* has illustrated these assumptions in the following diagram.

Group	O	A		B		AB
Agglutinogen	$\ddot{o}\ddot{o}$	$A\ddot{o}$	AA	$B\ddot{o}$	BB	AB
Agglutinins	$ab(\ddot{o})$	$(a)b(\ddot{o})$	$(a)b\ddot{o}$	$a(b)(\ddot{o})$	$a(b)\ddot{o}$	$(a)(b)\ddot{o}$

In this diagram it will be observed that the agglutinins involved in a reciprocal binding with the corresponding agglutinogens in the red cells are enclosed in parentheses. The reciprocal binding prevents autoagglutination. Conclusions concerning non-paternity are not in conflict in the two theories except where AB parents are involved. Reference to Table V shows that accord-

*Snyder: Blood Grouping in Relation to Clinical and Legal Medicine, Williams and Wilkins Co. Published by permission of Snyder and Williams and Wilkins Co.

ing to the von Dungern and Hirschfeld theory children of all types are possible in any matings which involve an AB parent. According to the Bernstein theory the following matings never result in O children: B x AB, AB x AB, A x AB. In O x AB matings A and B children may result but not O or AB children. While there is a general acceptance of Bernstein's hypothesis, Snyder says that it is not yet proved beyond all doubt. The student is referred to the studies of Ottenberg (1928) and Wiener (1930) for a more comprehensive discussion of these two as well as other theories under consideration at the present time.

Subgroups.—In 1911, von Dungern and Hirschfeld apparently demonstrated by means of agglutinin absorption experiments that individuals having the A factor fall into two subgroups, those that are purely A and those in which the A agglutininogen is linked with a second agglutininogen A'. Likewise, they apparently showed that there are two kinds of group B sera, those containing only the α agglutinins and the other containing the α agglutinin always associated with an α' agglutinin. Others* (see Landsteiner, 1928) have shown that many group O sera contain α' as well as α and β agglutinins.

Importance of Quantitative Difference.—The existence of subgroups for A has been confirmed by Schütze (1921), Coca and Klein (1923), Guthrie and Huck (1923) and Landsteiner and Witt (see Landsteiner, 1928). Lattes and Cavazutti (1924) are of the opinion that these results are all due to quantitative differences in the agglutinability of corpuscles rather than the existence of a third pair of agglutinins and agglutinogens. After carefully investigating the question raised by Lattes and Cavazutti, Landsteiner (1928) concludes that the evidence at hand warrants the assumption of α' -A' factors. He states, however, that quantitative variations, as suggested by Lattes and Cavazutti, may account for many blood differences reported in the literature. Table VI, showing the incidence of the third pair of agglutinating factors, is taken for the most part from Simson's (1926) detailed study with some rearrangement of data and an adoption of a uniform nomenclature. These subgroups are also designated as A₁ corresponding to A' and A₂ here called A, the corresponding agglutinins being α_1 and α_2 .

*See Supplementary References, p. 192, for papers by Wiener (1941).

TABLE VI
INCIDENCE OF OCCURRENCE OF α' AND A' FACTORS

JANSKY	GROUP MOSS	TOTAL NO. CROSS AGGLUTINATED	NO. OF SERA ABSORBED	AGGLUTININS IN SERA	AGGLUTINING REAGENTS IN RED CELLS	NO. OF EACH TYPE	PER CENT
I	IV	143	25	$\alpha\beta$ $\alpha\alpha'\beta$	O O	1 24	4 96
II	II	225		β β	A AA'	43 182	19 81
III	III	42	12	α $\alpha\alpha'$	B B	1 11	8 92
IV	I	11	—	— —	AB AA'B	2 9	18 82

PRESENCE OF SUBGROUPS DOES NOT INTERFERE WITH ROUTINE TYPING.—Since the α' agglutinin is almost always found associated with the α agglutinin in sera and the A' agglutinogen likewise associated with the A agglutinogen, it is evident that their existence does not invalidate any conclusions as to blood grouping based upon the postulates of Landsteiner. In other words, their presence cannot be detected by the routine method of typing, but is detected only by means of absorption experiments.

Irregular Agglutination.—Landsteiner and Levine (1929) have made an extensive study of "isoagglutinin reactions of human blood other than those defining the blood groups." They cite the observation of Thomsen (1928) that in five out of 3,500 bloods examined he definitely proved an absence of isoagglutinins from sera of bloods belonging to groups O, A, and B. In this series he observed 32 abnormal sera. Guthrie and Huck (1924-25) found one serum of group B which contained only an agglutinin for one subgroup of A. In 1926, Landsteiner and Levine noted two sera of bloods belonging to group AB that contained an α' agglutinin.

IMPORTANCE OF TEMPERATURE.—In later studies (1929) they used very sensitive tests by employing diluted blood (2.5 per cent) and temperatures of 20°, 25°, 30° and 37° C. Most of the abnormal reactions occurred at temperatures below 37° C. At the latter temperature the abnormal agglutination either did not occur or it disappeared when cells agglutinated at 20° C. were warmed to 37° C. Landsteiner and Levine state that "for several sera the upper limit of activity was at 30° C., for others at 25° or even 20° C." In all they examined 500 sera. They found three sera of group AB that contained α' agglutinin, two sera of group AB and one of group A that agglutinated intensely group O cells and to a lesser degree cells belonging to subgroup A₂. They further state that "in a total of about 500 sera examined there were at least 16 that gave reactions designated as +, i.e., about 3 per cent. Of 180 sera 12 or 6.6 per cent reacted weakly and 31 sera (about 17 per cent) showed traces of agglutination within the same group." They conclude that "one can speak of abnormally reacting sera but hardly of abnormal blood cells."

M, N, and P Factors of Human Blood.—In 1928, Landsteiner and Levine demonstrated, by means of immune sera, three new

agglutinable factors distributed among the four blood groups. They found at that time no normal agglutinins in human sera corresponding to these three factors which they designated as M, N, and P agglutinogens. Subsequent studies have shown the M factor to be strictly defined whereas the property P is not. In regard to the latter, they make the following statement, "thus the property P is not strictly defined like A, B, or M, but designates a group of related agglutinable factors." In regard to the N factor they say (July, 1929) that it, like P, shows fluctuation but not to the extent of the latter. Landsteiner and Levine (February, 1929) studied the distribution of these factors among the four blood groups of both white and colored individuals. They found the M agglutinin present in 80.9 per cent of white and 71.9 per cent of colored individuals examined. The distribution among the four blood groups is quite uniform. The N factor is likewise uniformly distributed among the groups and shows the same incidence in both colored and white individuals. Landsteiner and Levine found it present in 73.9 per cent of the white and 72.4 per cent of the colored individuals examined by them. In regard to the P factor they state, "The strongest reactions were almost four times as frequent in the colored as in the white individuals, whereas the weak reactions were much rarer in the former. Blood with negative reactions occurred only exceptionally among the colored."

Landsteiner and Levine (1928) have shown that M and N factors are inherited as Mendelian dominants, probably dependent upon a single pair of allelomorphous genes M and N. The resultant possible types are M, N, and MN. These factors occur as agglutinogens demonstrable only by immune serum. These immune sera are usually prepared by the injection of known M and N blood cells into rabbits. Recent work (Stuart, et al., 1936, 1937, 1939) has shown that rabbits, in order to produce potent antibodies, must not contain M factors in their tissues. Studies on the distribution of these factors show that M and N occur regularly in human cells but are independent of the A and B factors. They may be used in medico-legal work in the same way as the A and B factors. However, by the determination of M and N as well as A and B the average chances of proving nonpaternity are doubled.

Extra Agglutinin 1.—In July, 1929, Landsteiner and Levine called attention to an extra agglutinin which they found in two sera of group O, three of group A, and one of group B. This new agglutinin "gave reactions with bloods of various groups." In February, 1930, they report the results of further studies upon this new agglutinin and designate it as "extra agglutinin 1." They state that there is some apparent relationship between the agglutinin and the P factor.

In July, 1930, Nigg reported her studies of two unusual type B bloods. Both contained an agglutinin demonstrable at 25° C. for many type O as well as type B cells. Agglutination did not occur, however, at temperatures above 30° C. She concluded that these agglutinins were identical with the "extra agglutinin 1" of Landsteiner and Levine. Since then, Landsteiner and Levine (1931) have carried out extensive studies on "The Differentiation of a Type of Human Blood by Means of Normal Animal Serum." They conclude that this "extra agglutinin 1" is specific for the P factor. Thus it is evident that occasionally the serum of groups O, A, or B individuals may contain an extra agglutinin for an agglutigen P that has been previously discussed. When a serum of this kind is encountered and compatibility tests are done at room temperature, some confusion may result unless the phenomenon is understood.

Irregular Isoagglutination at Temperatures Below 37° C.—If one carries out agglutination tests using the sera and cells of different individuals and varies the temperature between 37° and 0° C., he will observe many individual variations not distinguishable at body temperature. The number of these interesting variations increases as the temperature is lowered. Many of the reactions are due to the presence of α^1 and α^2 agglutinins and their corresponding agglutinogens but others are not thoroughly understood.

AUTOAGGLUTININS.—Landsteiner and Levine (1926) and Landsteiner (1928) call attention to the frequent occurrence of autoagglutination at temperatures between 0° C. and 5° C. The autoagglutinins can be absorbed by the homologous red cells at 0° C. and after washing with saline at the same temperature, the agglutinins can be recovered by warming the suspension to 37° C. or even less. Marked autoagglutination has been observed in

a number of pathological conditions such as hypertrophic cirrhosis of the liver, hemolytic jaundice, paroxysmal hemoglobinuria, Raynaud's disease, and trypanosomiasis in man and animals.

COLD AGGLUTININS.—The term “cold agglutinins” is used by Li Chen-Pien (1926) and others to indicate autoagglutinins, but Landsteiner (1928) considers any hemagglutinins that bring about real agglutination only at low temperatures of either the individual's own corpuscles or those of others of the same species, “cold agglutinins.” On the other hand, Snyder differentiates between autoagglutinins and cold agglutinins for he says, “When the individual's red cells are agglutinated under such conditions (0° - 5° C.) by his own serum, the reaction is spoken of as ‘auto-agglutination.’ When the agglutination occurs due to serum of another individual, the reaction is known as ‘cold agglutination.’ These two reactions are certainly related and may be identical.” Landsteiner's views seem to be in harmony with the term “cold agglutinins” and are hence recommended to the student as perhaps the most satisfactory.

PSEUDOAGGLUTINATION.—The phenomenon of pseudoagglutination occurs at low temperatures and may be intensified at 37° C. It is due to rouleaux formation in which the red cells adhere together like piles of coins. The mechanism of rouleaux formation does not involve agglutinins and hence is an entirely different phenomenon from agglutination. Snyder (1929) says that the tendency for rouleaux formation is frequently increased in “rheumatic fever, tuberculosis, pneumonia, cardiac diseases and in certain physiologic conditions such as menstruation and pregnancy” or any condition showing an increase in the rate of sedimentation of the red cells.

Inhibition of Rouleaux Formation.—Snyder cites the work of Lattes (1924) and Falgairolle (1926) who found that rouleaux formation is inhibited by adding lecithin and other substances to the serum. Snyder (1929) states that one part of kaolin to three parts of serum will also inhibit rouleaux formation. He also suggests the use of formalin and hypotonic salt solution. Shattock (1900) noted that diluting the serum with saline prevented pseudoagglutination. Rouleaux formation and true agglutination of red cells are illustrated in Figs. 8 and 9 respectively.

Nature and Distribution of Red Cell Haptens.—In his study of cellular antigens Landsteiner (1936) discusses the nature of the haptens which determine blood groups. They are alcohol-soluble substances, probably lipoidal in nature, one for the A and a second for the B factor. In his opinion they are somewhat analogous in nature to Forssman's antigen. Schiff and Adelsberger (1924) conclude that the A factor in human cells is heterophile in nature

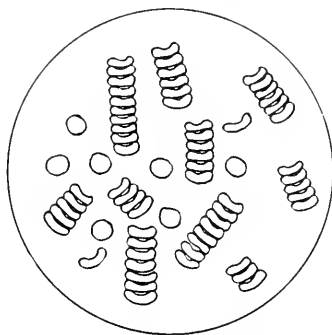


Fig. 8.—Pseudoagglutination or rouleaux formation of red cells. (Microscopic.)

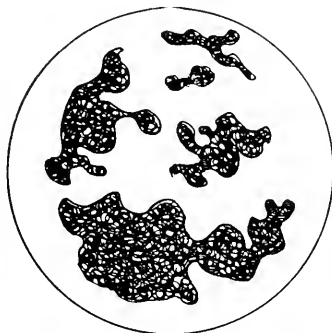


Fig. 9.—True agglutination of red cells. (Microscopic.)

since they found group reactions for sheep cells and those of group A (human). It is also of interest to note that the normal antish sheep hemolysins frequently found in human blood are regarded as heterophile antibodies. Landsteiner (1928) suggests that perhaps the antigens of the red cell may have a mosaic structure, since this concept apparently harmonizes with observed phenomena, such as the ease with which antibodies can be fractionated by absorption. Recent work by Landsteiner (1936) and

Schiff and Adelsberger (1924) has suggested that the group specific A hapten is a lipoid-carbohydrate complex owing its specific reactions to carbohydrate groups.

Friedenreich (1939) has demonstrated that group specific substances identical or similar to the group A substance in human red cells are widely distributed in human tissues and secretions. In addition, similar group A substances are found in nature from such diverse sources as horse and bovine saliva and certain pneumococci.

Summary.—It is hoped that the following partial summary of material in this chapter may be of help to the student.

In 1901, Landsteiner discovered three of the four recognized blood groups within the human species and postulated two agglutinogens A and B and two agglutinins α and β . He also suggested the law of reciprocal relationships according to which, when an agglutinogen is present in the red cells the corresponding agglutinin is absent from the serum, and conversely, when the agglutinin is present in the serum, the corresponding agglutinogen is absent from the red cells of that particular blood.

The following year DeCastello and Sturli described four examples of the fourth group. They considered a classification identical with that of Moss (1909-10), but did not feel warranted in recommending its adoption. Since the four examples of what they termed group 1 (AB) were all infants, they thought perhaps the latter had not developed a stable group. It is interesting to note that DeCastello and Sturli conceived that some type of what has been more recently called reciprocal binding may exist between an agglutinogen and agglutinin which would prevent auto-agglutination. The classifications suggested by Jansky (1907) and Moss are given and compared with a new classification being used quite generally in the literature and based upon the agglutinogen content of the cells. The agglutinogens are present in the red cells at birth. The agglutinins may be present then, or make their appearance at some time during the first four years of life. When once established, the group remains stable except, perhaps, for quantitative variations in titer of agglutinins.

The racial distribution of the blood groups is discussed briefly in connection with the two-factor and the triple allelomorph theory.

The latter, proposed by Bernstein, seems to fit the data at hand. For practical purposes in blood typing the assumption made by Landsteiner that there are two agglutinogens and two corresponding agglutinins is quite satisfactory. Evidence is presented indicating that the blood groups are inherited and that in some, but not all, cases of disputed paternity nonpaternity may be established for certain individuals.

Attention is also called to the existence of subgroups and to the fact that their existence does not interfere with ordinary blood typing. The new agglutinable factors, M, N and P were discovered by Landsteiner et al. Normal agglutinins for M and perhaps for N have not been observed, but a few individuals, representing practically all the groups, have been found whose blood contains an agglutinin for P. This is the same as "extra agglutinin 1" of Landsteiner. Fortunately these reactions disappear, as a rule, at temperatures above 30° C. Where typing is done at room temperature they may be very rarely encountered. The agglutinable factors present in the blood of lower animals and of the anthropoid apes are also mentioned in connection with their biological significance.

Various irregular agglutinations are discussed and a definition of "cold agglutinins" is given.

The antigenic factors of the red cells are discussed. Landsteiner's views concerning these are developed. Cellular antigens are complex. Two types of specificity exist, one for the species and a second indicating specific differences within a species. The species specific antigens of the red cell are represented by isophile hemolysins, a nonspecies specific antigen by the heterophile substances. Differences within a species are represented by the agglutinable factors A and B for human blood. The antigenic structure can be thought of as resembling a mosaic. Chemically speaking, the red cell antigens are made up of one or more protein structures and a number of peculiar lipoid-carbohydrated complexes or haptens. The various haptens such as those for the A factor or the B factor confer definite specificity upon an antigen. The A substances seem to be widely distributed in the tissues of man and animals and even in bacteria.

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CHAPTER XI

NATURE, FORMATION, ACTION AND MEASUREMENT OF ANTIBODIES

Introduction.—When one reviews the basic facts that have been established beyond reasonable doubt about the plasma proteins, he discovers that an important one is missing, since no one knows with certainty the source of these plasma proteins. The immunologist usually defibrinates the blood and works with the serum proteins which remain. These represent a virtual spectrum of colloids ranging from the water-insoluble and soluble euglobulins through the pseudoglobulins to the water-soluble albumins. In the circulating plasma they probably represent a very complex arrangement of colloidal particles varying in size, amount of bound water, chemical content and structure, electrical charges, etc., and play many roles in the body's economy.

By adding sodium sulphate to the extent of 13, 17 and 21 per cent to different portions of serum, Howe preecipitated three fractions of proteins which are called euglobulins, pseudoglobulin 1 and pseudoglobulin 2, respectively. The albumins remained in solution but are preecipitated by higher concentrations of sodium sulphate. Others have separated the serum proteins into these and additional fractions by other methods. The most recent and perhaps the most successful one is that of Tiselius, who separates proteins into different fractions according to their rate of movement between two electrodes.

Antibodies.—In previous chapters attention has been called to research indicating that the blood plasma is virtually a reservoir of *traces* of reacting substances or groups having more or less specific affinities for many red cells of the same and different species, for various kinds of bacteria, lipoids or lipid-carbohydrate complexes and various bacterial polysaccharides. These specific reacting factors are called *natural antibodies* and the substances for which they have specific affinities are called either *antigens* or *partial antigens* (haptens as Landsteiner calls them). They are real antigens if they can stimulate some animal body to produce specific antibodies and partial antigens or haptens if

they react well with antibodies but either do not stimulate their production or are very weak stimulators.

Just why these antibodies are present in normal blood is not definitely known. Some of them, like the isohemagglutinins, heterohemagglutinins and lysins, are apparently normal physiological products and it is possible that part of the antibodies for infectious agents of various kinds likewise are normal physiological products. Sutliff and Davies (1937) studied nine infants over a five-month period in an attempt to see whether there was any relationship between the strain of pneumococcus in the nasopharynx and the type of antibody in the blood. They report that no relationship could be noted although the pneumococidal action of whole blood against Type II pneumococcus increased in three infants and for Type III pneumococcus in one infant. They suggest the advisability of investigating the possible physiological origin in future work.

It is also possible that an undetermined amount of the so-called normal antibodies for bacteria may owe their presence to unrecognized infection with the organism corresponding to the antibody. The presence of the bacteria in the body due to unrecognized or recognized infection or as a result of vaccination would represent antigenic stimulation. Presumably this would result in antibody production.

Antigens.—As a prelude to any further discussion of antibodies it would seem desirable to recall some of the pertinent facts about antigens that have either been mentioned or that appear in later chapters. Both the colloidal state and solubility in the body fluids seem to be properties of all true antigens.

For a long time it was thought that only complete proteins could stimulate specific antibodies and therefore be regarded as true antigens. It is now quite generally agreed that the nitrogen containing polysaccharide specific for Type I pneumococcus and a few other bacterial polysaccharides and perhaps a few lipid-carbohydrate complexes from cells and tissues can stimulate specific antibodies when injected into certain animals. It was likewise thought for a long time that antibodies would react only with complete proteins, but Zinsser and Parker (1923) discovered non-protein substances (haptens) in bacteria that reacted with bac-

terial antibodies. Heidelberger and Avery found that such substances in pneumococci are polysaccharides. It is now known that high molecular carbohydrates are important as determinants of specificity of many bacterial agglutinin and precipitin reactions (Landsteiner, 1936, p. 44) and Schiff and Adelsberger* seem to have shown that human red cell haptens are either complex polysaccharides or lipid-carbohydrate combinations. Landsteiner and his associates have shown that a wide variety of substances may function as haptens (see Chapters XVII and XVIII).

In a later chapter attention is called to the conception of Ehrlich supported by experimental proof of Wells and Osborne that antigenic specificity depends upon the chemical constitution of the antigen. Landsteiner (1936, p. 73-74) calls attention to the possibility of a single antigen having several reacting groups and that, therefore, a number of antibodies may be formed for one antigen. He and van der Scheer (1938) have also shown that separate antibodies may be formed for portions of an antigenic molecule. It is apparently established that cells contain many antigenic factors, and it is thought that the cell antigens present a mosaic structure. It is apparent that the surface antigenic factors will play a more important role in serological reactions than antigenic factors buried beneath the surface. Their potentialities may be masked. For a more extensive discussion of the complex nature of antibodies the student is referred to a paper by Marrack and Carpenter (1938).

Nature and Origin of Antibodies.—The immunologists have found that the antibodies are precipitated with the euglobulin or pseudoglobulin fractions of serum. According to Landsteiner (1936, p. 94) it is not yet decided whether antibodies are proteins or substances intimately bound to the proteins. There is strong evidence indicating that they are protein in nature and therefore are frequently called *antibody-globulins* or *immune-globulins*. They unite readily with their antigen at 0° C., are not dialyzable, and resist drying for long periods of time.

Fahey and Green (1938) obtained from normal horse serum *three* water-insoluble proteins (euglobulins) by isoelectric precipitation in a very low salt concentration. They named these P_I, P_{II}, and P_{III} fractions. In a second communication Green, McKhann, Kapnick and Fahey applied the same methods to a study of frac-

*See discussion by Landsteiner (supplementary references).

tionation of the globulins of Types 1 and 2 antipneumococcal horse serum. They describe a new fraction of water-insoluble globulin which contains most of the antibody. They named the fraction P_{IV}. It seems to be made at the expense of P_{II} since the latter had almost entirely disappeared when P_{IV} appeared. The authors suggest that the new antibody globulin may be a modified form of the normal globulin fraction P_{II}.

Ando, Takeda and Hamano (1937, 1938) report isolating two fractions (A and B) of immune rabbit serum that contain antibodies. Fraction A is a water-soluble globulin and contains antibodies such as the antitoxins and the shiga-dysentery and perhaps Paratyphosus B. antipolysaccharide antibodies. Fraction B is water *insoluble* and contains antibodies for pneumococci, *E. typhosa*, *P. pestis* and many other bacteria. They call attention to the fact that these antibodies characterizing one fraction may be found to some extent in the other. We have noted, in unpublished work, that while antibodies were present in either the euglobulin or pseudoglobulin I fraction they were found predominating first in one and then in the other. Perhaps imperfect methods of separation are partly responsible for these results.

Horsfall and Goodner (1935, 1936) have reported interesting differences in antipneumococcal sera obtained from different animal species. They were able to divide the ten Type I antipneumococcal sera from the ten animal species studied by them into two groups. In group one, of which the horse-antiserum is a prototype, the antibody contains the lipoid *lecithin*, the presence of which seems to be necessary for agglutination or precipitation while the antibody of the rabbit, the prototype of group two, contains the lipoid *cephalin* which is essential for agglutination. They found the antibodies from which the essential lipid was removed would nevertheless combine with its antigen but neither agglutination nor precipitation would follow. Furthermore they found that while the antibodies of group one combined with the corresponding pneumococcal polysaccharide, the resulting complex fails to fix complement. On the other hand, the complex resulting from the union of antibodies of the second group with pneumococcus polysaccharide fixes complement.

Another interesting difference is in the immunological response the two groups of animals give to the injection of Type I pneumo-

coccal polysaccharide. The animals in group one respond with antibody production while those of group two give no antibody-response to its presence in a *free state* in the body. The sera comprising group one are horse, man, mouse, cat, dog and goat, while those of group two are rabbit, guinea pig, rat, and sheep.

The same authors (Horsfall and Goodner) have also shown that the antibody molecule in horse antipneumococcus serum is larger than the corresponding one in rabbit pneumococcus immune serum. This difference in size of the antibody molecule probably explains, in part at least, why the antiserum obtained from rabbits is more effective in the treatment of pneumonia than antiserum from the horse. Presumably the rabbit serum antibody molecule can diffuse more readily into the tissues. Another difference between antibody from horse and that from the rabbit is observed in the Quellung reaction where the antibody from the horse fails to give the reaction in contrast with the positive results obtained with antibody from the rabbit. According to Chase and Landsteiner (1939) the molecular weights of Types I and III pneumococcal antibodies have been determined in Svedberg's laboratory to be 930,000 for horse, cow and pig and 157,000 for the rabbit and monkey.

Goodner and Horsfall (1937) have also shown that antipneumococcus horse serum contains at least three antibodies which precipitate the specific polysaccharide but differ in their ability to protect mice. They employed Heidelberger's method of separating antibodies from immune precipitates in hypertonic salt solution. By employing 10 per cent sodium chloride they separated two fractions of antibody. One of these antibodies was a water-soluble pseudoglobulin-like substance, P, which had low protective capacity and a second water-insoluble (euglobulin-like) antibody, E, which exhibited more protective power but formed precipitates less rapidly than P. Chase and Landsteiner suggest that these antibodies may both be leicthoproteins.

ORIGIN OF ANTIBODIES.—Buchner originally conceived of antibodies as being formed from the antigens injected but his theory soon gave way to the "side chain theory" of Ehrlich. Recently Manwaring and others have attempted to revive Buchner's theory although modifying it to some extent. Heidelberger (1932) in discussing Manwaring's theory cites a statement by Doerr "that

arsenic in an atoxyl-azo antigen leads to an arsenic-free antibody, although the arsenic acid radical determines the specificity." This is further borne out by the work of Heidelberger and Kendall, who found that R-salt-azobenzidine-azo-egg albumin gives rise to colorless antibodies although the colored part of the antigen is the portion determining specificity.

Heidelberger further calls attention to the failure of quantitative studies to support Manwaring's views since he says, "As much as eight mg. of circulating antibody may be produced per milligram of antigen injected, and since this is only a portion of the total antibody produced, the amount seems inconsistent with the idea that specific antigen fragments are present, though the evidence is not conclusive." He then cites the work of Hooker and Boyd and also of Topley, who disagree with Manwaring. In a later paper Heidelberger, Kendall, and Soo Hoo (1933) state that they obtained a total response for the rabbit of over 100 mg. of circulating antibody for every milligram of antigen injected.

Buchner's original theory was soon supplanted by Ehrlich's side-chain theory. He conceived of antibodies as chemical food receptors produced by the tissue cells in excess as a result of stimulation resulting from injury or demand. Since his theory is only of historical interest it will not be discussed.

Heidelberger (1932) reviews the work of Breinl and Haurowitz who conclude that the presence of antigen in the tissues "disturbs the mechanism of globulin synthesis, probably in the amino-acid-peptid stage, modifying the method of union or the spatial relations of the globulin components so that a new globulin, an *antibody*, is formed which reacts specifically with the antigen by virtue of the distortion caused by the presence of the antigen at the moment of synthesis, for if the antigen can only affect amino acids having affinity for it, these should retain that affinity after their synthesis into globulin."

Sabin (1939) suggests that antibodies represent proteins cast off by the clasmotocytes. While no one seems to know positively how they are formed, there is a great deal of evidence indicating they are formed by cells of the reticulo-endothelial system.

UNITARIAN THEORY OF ANTIBODIES.—Zinsser (1939) says that this theory does not imply that only one antibody will be produced against a complex cellular antigen. He says, however, that

according to the unitarian theory a single antigen, in a pure state, would stimulate the formation of one variety of antibody capable of uniting with the antigen. This union of antigen and antibody could result in agglutination, precipitation, complement fixation, bactericidal phenomena, opsonization or anaphylactic sensitization depending upon the physical state of the antigen itself, "the nature of the cooperative substances (alexin, leucocytes, tissue cells), and by the environmental conditions under which the observations are made." Delves (1937) as well as Zinsser, Enders and Fothergill (1939, pp. 175-178) cite considerable experimental evidence in support of this theory.

While it has been apparently established that an antigen may give rise to more than one antibody, yet it is conceivable that any one of the antibodies may serve some or all of the various capacities mentioned by Zinsser. It is possible, as suggested by the work of Horsfall and Goodner mentioned earlier in this chapter, that antibodies from some species of animals may not promote complement fixation although they lead to precipitation. With these points in mind we are accepting the general tenets of his Unitarian theory until more evidence against it is available.

MECHANISM OF ANTIGEN-ANTIBODY UNION.—There are at least two theories explaining antigen-antibody union that are being discussed at present. According to the *film hypothesis* supported by Eagle, Mudd and others, the antibody is specifically adsorbed to the antigen forming a partial or complete film on the surface. The antibody globulin becomes insoluble in saline (denatured) as a result of this union with antigen. Eagle suggests that the specificity of the antibody globulin is due to its hydrophilic (water-loving) groups which become attached to the antigen, thus orienting the hydrophobic ends of the molecules outward toward the water which accounts for the denaturing of the antibody protein. Marrack, on the other hand, cites the quantitative studies of Heidelberger and Kendall as offering evidence against the film hypothesis. He thinks the antibody molecule combines as a closely packed group of molecules and not as a film. As a working theory we are tentatively accepting the film hypothesis as outlined by Eagle and also Mudd.

There are also two lines of thought relative to the mechanism of agglutination and precipitation which may result from antigen-

antibody union. The one theory first suggested by Bordet and supported by experimental data outlined in Chapter XII assumes that the agglutination or precipitation is due to the reduction in repelling force between the cells or colloidal particles and the existence of cohesive properties of the sensitized antigen. According to this theory these physical changes in state of aggregation are secondary phenomena in which chemical forces do not play a role. The second theory, supported by experimental work of Heidelberger and Kabat (1937), states that chemical forces play a role in the second stage which is agglutination of cells or precipitation of colloidal particles. Opposition to this latter concept is offered by Hooker and Boyd (1938) and Eagle (1938). The explanation we offer in Chapter XII is largely in harmony with the explanation offered by Bordet, Eagle, Hooker and Boyd and others. We realize there is considerable evidence in support of the opposing view but until there is a general acceptance of it we will hold to the older theory.

Method of Measuring Antibodies.—

ANTITOXINS.—Ehrlich suggested a method of standardizing antitoxin, which, while used to some extent, is being superseded by the Ramon flocculation technique. Ehrlich was working with diphtheria toxin and determined the smallest amount that would kill a 250 gram guinea pig in four or five days. This he called a minimum lethal dose or an M.L.D. He then measured out 100 M.L.D.'s and called that amount an L_0 dose of toxin. The amount of antitoxin that just neutralized this he called a unit of antitoxin. This amount corresponds to what is now called an "*old unit*." He then determined the least amount of toxin that, when mixed with an "*old*" unit of antitoxin, would kill a 250 gram guinea pig in four to five days. This amount of toxin he called the L_+ dose. The least amount of antitoxin which protects the guinea pig against the L_+ dose of toxin is the "*new unit*." In Chapter XIV the method of titrating antitoxin by the Ramon flocculation technique is given. Eaton (1936) discusses the flocculation reaction with purified diphtheria toxin. This method is also applied to standardizing tetanus and other antitoxins.

UNIT OF TETANUS ANTITOXIN.—The American immunity unit of tetanus antitoxin has been defined as "ten times the least

quantity of antitetanus serum necessary to save the life of a 350 gm. guinea pig for ninety-six hours against the official dose of standard toxin furnished by the Hygienic Laboratory of the Public Health and Marine Hospital Service." Compared to a unit of diphtheria antitoxin it has slightly more than ten times the protective power. The method of standardizing tetanus antitoxin was suggested by Rosenau and Anderson (1907). As mentioned above, the Ramon technique is now used extensively.

Scarlet fever antitoxin is measured in terms of its protective value for skin test doses of toxin in accordance with the work of Dick and Dick (1924).

AGGLUTININS.—In determining the titer of a clumping or agglutinating serum, as, e.g., one that agglutinates *E. typhosa*, the antigen is not diluted, as in the precipitin ring test. On the contrary, a series of serial dilutions of the immune serum is made. A number of small test tubes are placed in a rack and to each is added a uniform and measured amount of the various dilutions and an equal amount of a standard turbid suspension of the antigen, e.g., *E. typhosa*. After shaking and incubating, notation is made of the highest final dilution of the immune serum containing the antibody that gives perceptible clumping, and this is the titer of the immune serum. Titers as high as 1:5,000 or 1:10,000 are readily produced in rabbits by vaccination. The suspension of bacteria used in agglutination work is usually diluted to match a known turbidity standard.

PRECIPITINS.—The strength or titer of a precipitating immune serum is commonly described in terms of the highest dilution of the *antigen* that gives a perceptible ring precipitate when stratified over the undiluted immune serum in a small test tube. Titers as high as 1:1,000,000 have been produced.

Cannon and Marshall (1940) have called attention to an old but *more rational method* of determining the titer of precipitating antibodies. They prepare a standard suspension of collodion particles, film them with antigen and mix an equal volume of the filmed particles with a corresponding volume of serial dilutions of the antibody as in an agglutination test. After incubation for 30 minutes at 37° C. the tubes are centrifuged at low speed to bring together antigen-antibody coated particles. The contents of the tubes are then resuspended and examined for agglutination.

The highest dilution of antibody giving perceptible agglutination of the particles filmed with antigen is the titer of the precipitin serum.

BACTERIOTROPINS.—The content of bacteriotropins in a patient's serum is estimated by mixing equal parts of the serum, bacterial suspensions and a suspension of leucocytes, incubating and determining, by microscopic examination of stained smears of the incubated mixture, the average number of bacteria ingested per leucocyte. A normal serum is treated in like manner and the average number of bacteria engulfed per leucocyte is determined. If the mixture containing patient's serum showed, e.g., four bacteria phagocytized per white cell, and two bacteria per white cell in the mixture containing normal serum, the opsonic index would be $\frac{4}{2}$ —or 2. In this example the patient's serum would cause twice as much phagocytosis of the organism used as normal serum.

ANTIAGGRESSINS.—There is considerable doubt in the minds of many, of the existence of such an antibody as an antiaggressin. According to Weil and others it is present in the albumin fraction of the serum. The antiaggressin content is estimated from the amount of serum that protects guinea pigs against a predetermined number of minimum fatal doses of the organisms.

HEMOLYSINS.—In order to measure the strength, i.e., determine the titer of an hemolytic immune serum, one must adopt definitions of what constitutes a unit of each of the three constituents used, i.e., red cell suspension, sensitizer and complement. Kolmer's definition of each of these may be expressed as follows:

Kolmer has defined a *unit of red cell suspension* as one-half cubic centimeter of a 2 per cent suspension of washed sheep red cells in normal saline. Washed packed cells, obtained by centrifuging defibrinated sheep blood and repeatedly resuspending in saline with subsequent centrifugalization, constitutes a 100 per cent suspension. From the final packed cell sediment a 2 per cent suspension is prepared. Other authorities have arbitrarily adopted different kinds of red cells as well as different volumes and weights of suspensions in their definitions of what each regarded as a unit. These will be mentioned in the chapter on Complement Fixation.

A *hemolytic amboceptor or sensitizer unit*, according to Kolmer, is 0.5 c.c. of the highest dilution of immune serum that will

sensitize one unit of red cells so that they will be completely hemolyzed by 0.30 c.c. of a 1:30 dilution of guinea pig complement after incubation for 1 hr. in a 37° C. water bath.

A *unit of complement* is the least amount of a 1:30 dilution of complement that will completely hemolyze one unit of red cells sensitized by two units of hemolysin. Kolmer always titrates complement in the presence of a test dose of antigen.

METHOD OF TITRATING HEMOLYSIN.—In order to determine experimentally the amount of immune serum that contains a unit of hemolysin or to ascertain the amount of normal guinea pig serum that contains one unit of complement, certain standards have to be arbitrarily adopted. Kolmer, who has carried out an extensive investigation of various methods used to titrate hemolysin and complement, recommends that after all reagents are added their final volume shall be 3.0 c.c. in each tube. He has adopted 0.5 c.c. of a 2 per cent suspension of washed sheep red cells as a unit for the red cell suspension and normal saline containing 0.1 gm. of magnesium sulphate per liter as a diluent. He makes up a series of dilutions of the immune serum using a separate test tube for each dilution and carefully labels each tube indicating the dilution it contains. These dilutions are usually as follows: 1:1,000, 1:2,000, 1:3,000, 1:4,000, 1:5,000, 1:6,000, 1:8,000, 1:10,000, 1:12,000, 1:16,000. He then dilutes fresh normal guinea pig serum (complement) 1:30 by adding one part of complement to twenty-nine parts of normal saline. After this he places ten Wassermann test tubes in a rack and adds the reagents according to the following protocol:

HEMOLYSIN TITRATION

TUBE NO.	HEMOLYSIN, 0.5 C.C.	COMPLEMENT 1:30 DILUTION C.C.	COLD SALINE SOLUTION C.C.	2% RED CELL SUSPENSION C.C.
1	1: 1,000	0.3	1.7	0.5
2	1: 2,000	0.3	1.7	0.5
3	1: 3,000	0.3	1.7	0.5
4	1: 4,000	0.3	1.7	0.5
5	1: 5,000	0.3	1.7	0.5
6	1: 6,000	0.3	1.7	0.5
7	1: 8,000	0.3	1.7	0.5
8	1:10,000	0.3	1.7	0.5
9	1:12,000	0.3	1.7	0.5
10	1:16,000	0.3	1.7	0.5

The contents of each tube should be mixed and incubated in a water bath at 37° C. for 1 hour. The unit is the highest dilution of hemolysin that gives complete hemolysis. Plate IV shows such a titration in which tube 5 (1:5,000) contains 0.5 c.c. of the highest dilution giving complete hemolysis. The unit or titer in this case is 0.5 c.c. of a 1:5,000 dilution.

COMPLEMENT TITRATION.—For complement titration Kolmer uses a 1:30 dilution of complement and always titrates this in the presence of a test dose of antigen contained in 0.5 c.c. of saline. He makes a point of using only cold saline in the titration. The titration requires 9 tubes for the varying amounts of complement shown in Plate IV, 2 and a tenth tube containing only saline and red cells (not shown in the plate). He sets up the complement titration according to the following protocol:

COMPLEMENT TITRATION

TUBE	COMPLEMENT C.C. (1:30)	ANTIGEN DOSE C.C.	SALINE SOLUTION C.C.	Water bath 37° C. for 1 hour	HEMOLYSIN C.C. (2 UNITS)	2 PER CENT RED CELL SUS- PENSION C.C.	Water bath 37° C. for 1 hour
1	0.1	0.5	1.4		0.5	0.5	
2	0.15	0.5	1.4		0.5	0.5	
3	0.20	0.5	1.3		0.5	0.5	
4	0.25	0.5	1.3		0.5	0.5	
5	0.30	0.5	1.2		0.5	0.5	
6	0.35	0.5	1.2		0.5	0.5	
7	0.40	0.5	1.1		0.5	0.5	
8	0.45	0.5	1.1		0.5	0.5	
9	0.50	0.5	1.0		0.5	0.5	
10	0.0	0.0	2.5		0.0	0.5	

UNIT OF COMPLEMENT.—The tube containing the *least* amount of complement that shows complete hemolysis is said to contain one *exact unit* of complement. In the illustration (Plate IV, 2) tube four contains the least amount of complement in which complete hemolysis results. In this example the *exact unit* is 0.25 c.c. of a 1:30 dilution. Kolmer defines a *full unit* as 0.05 c.c. more than an exact unit. This would be contained in tube five to which 0.30 c.c. of the 1:30 dilution of complement had been added.

The method of antigenic titration used by Kolmer and the various standards he has set up will be discussed in the chapter on complement fixation.

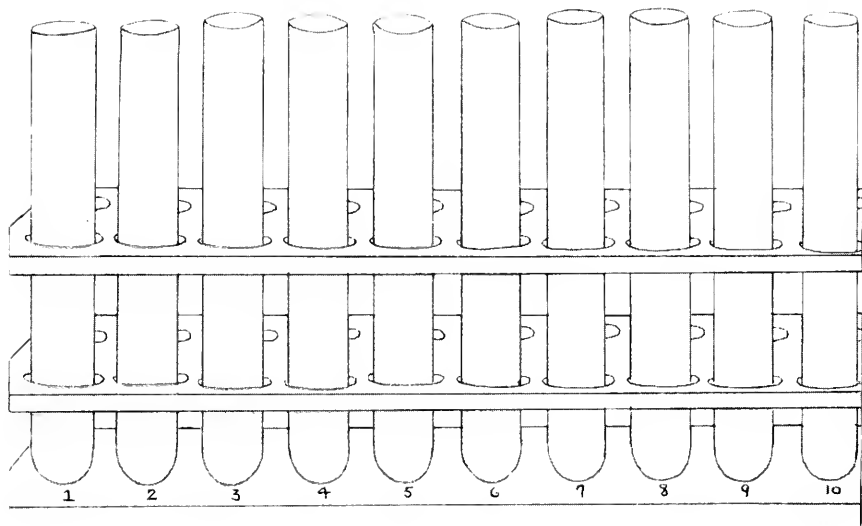


Fig. 1.—Tube 5 contains 1 unit of amboceptor.

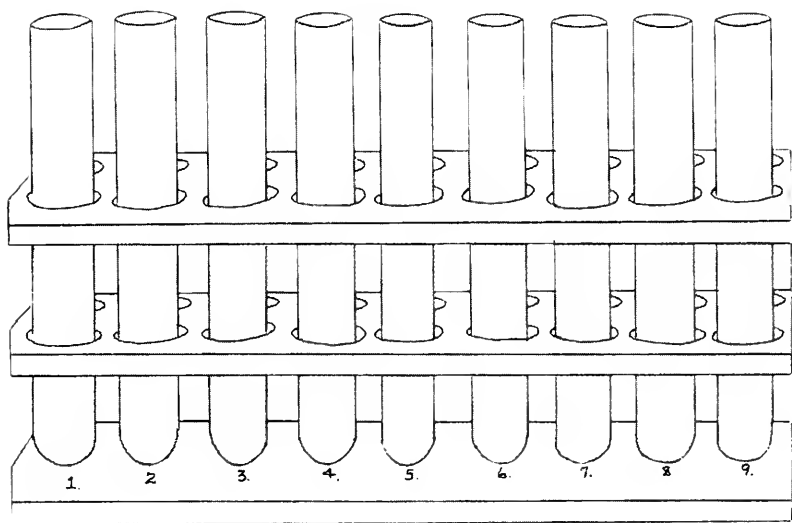


Fig. 2.—Tube 4 contains 1 unit of complement. Tube 5 contains 1 full unit of complement.

PLATE IV.—TITRATION OF HEMOLYTIC AMBOCEPTOR AND COMPLEMENT.

The reason for describing Kolmer's technique and omitting others is that the committee on Adherence to Conventional Technique in the Performance of Reliable Serologic Tests for Syphilis, appointed, I believe, by the Surgeon General, has approved the Kolmer complement fixation technique. For this reason it is being used, or is coming into use, in control and local laboratories throughout the United States. It is hoped that the student will realize that many of the requirements laid down by Kolmer are empirical ones while the principles underlying the tests are basic. It is true that empirical requirements such as the order of mixing, time of incubation, dilutions of amboceptor and complement used in titration, volume of fluid containing a unit or test dose, the unit of red cells, the temperature and time of inactivation, etc., all are based upon good reasons mostly for economy of time, reagents or for convenience. A good standard technique could be set up in which either slight or marked variation from Kolmer's would be used and results obtained that would be equally as accurate. Unfortunately there are bad modifications as well as good ones and it seems that in the interest of good serology it is best to recommend one good uniform technique as has been done by the committee. The *basic things* in all of these tests are the nature of the antigens, the laws governing the union of antigen and antibody, the binding of complement, the lysis of cells and the physical and chemical constitution of the reagents employed. These are not empirical things.

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CHAPTER XII

MECHANISM OF ANTIGEN-ANTIBODY REACTIONS CELLULAR AGGLUTINATION

Period of Discovery and Early Investigations.—The period of discovery of the phenomenon of bacterial agglutination by immune serum began with Charrin and Roger (1889) and ended with Bordet (1895). The next year the value of the reaction in diagnosis and its relative specificity was described by Gruber, Gruber and Durham, Widal, Grünbaum and others.

GRUBER'S THEORY OF AGGLUTINATION (1896).—Gruber suggested the name "agglutination" for the phenomenon and offered a theory explaining it. He believed that the clumping was caused by the action of agglutinin (antibody) upon the bacterial cell membrane whereby the latter became more viscous. In his opinion this change in the membrane caused the bacteria to stick together. This was really the first suggestion that "cohesive forces" might be a factor as shown recently by Northrop and De Kruif (1922).

BORDET'S EARLY WORK.—It is quite probable that all of this work was inspired by Bordet's observation (1895) of the phenomenon of agglutination while he was engaged upon the study of the phenomenon of Pfeiffer. The next year (1896) he turned his attention to the mechanism of agglutination and published the results of his experimental work two years later (1898).

DISCOVERY OF PRECIPITINS.—In the meantime, Kraus (1897) made an important discovery that has directly or indirectly influenced our conceptions of antigen-antibody reactions in general. He observed that a specific precipitate was formed when cholera-immune serum was mixed with filtrates from an old broth culture of the vibrio of Asiatic cholera. This was the first work done on precipitins. Two years later Tchistovitch noted that the blood serum of rabbits vaccinated against horse serum yielded a specific precipitate when mixed with horse serum. Similar results were obtained when eel serum was used as an antigen.

DINEUR'S HYPOTHESIS OF AGGLUTINATION.—Following Kraus's discovery and prior to Bordet's publication, Dineur (1898) ad-

vanced an hypothesis which was based upon Kraus's work but was really the beginning of our knowledge of flagellar agglutinins although Gruber had suggested that flagella might be important. Dineur conceived of the agglutination reaction as due to the formation of an "adhesive" substance on the flagella with the subsequent interlacing of the latter.

BORDET'S OBJECTIONS TO CURRENT THEORIES.—Bordet carefully considered Gruber's hypothesis that bacteria were agglutinated because the agglutinin caused them to become sticky. He could understand how changes in the cell membrane might make them "adhere" when they came in contact with each other, but this theory did not explain why they came together. In fact, there was no experimental evidence to show that a change in the membrane occurred. He knew from experience that hemagglutinins were developed in animals when red cells were injected and he watched the agglutination of red cells by immune serum under the microscope and could see no change in the membrane. Since red cells were agglutinated by their immune serum and since they did not possess flagella, he felt that flagella were not necessary factors in the phenomenon of agglutination, as suggested by others. Besides, Widal had shown that formalized cultures were agglutinated as readily as living suspensions and this he regarded as additional evidence. He then compared hemagglutination, bacterial agglutination, serum precipitation, and bacterial protein precipitation by their respective immune sera and saw that while they differed in that each antigen had in the beginning combined with its specific antibody, yet the end-result was quite similar; i.e., clumping or precipitation.

BORDET'S TWO-PHASE THEORY.—Thus Bordet considered two steps or phases in immunological reactions of this kind: First, one which involves specificity, the union of antigen and antibody, and second, a common mechanism of agglutination or precipitation. It was this second phase, involving a mechanism of agglutination common to all, that he set about investigating. At the beginning of his work he was very much impressed with Duclaux's theories as to the mechanism of coagulation and his conclusion that agglutination is a phenomenon of coagulation.

BORDET'S DEFINITION.—This led Bordet* to adopt the following definition for agglutination, "It is the union into masses of organized scattered particles by some peculiar influence that changes the properties of molecular adhesion." "The agglutination of bacteria is due to a change in molecular adhesion between the bodies of the bacilli and the surrounding fluid."

BORDET'S EXPERIMENTS ON EFFECT OF ELECTROLYTES ON CHOLERA VIBRIOS.—At that time it was apparently well known that clay forms a fine, homogeneous emulsion in distilled water, but that agglutination and rapid precipitation of the particles occurs when a small amount of sodium chloride is added. This suggested an experimental approach to the study of bacterial agglutination. Bordet made a suspension in salt solution using 24-hour-old cultures of *Microspira comma* and 10 c.c. of saline to each culture. To this homogeneous suspension he added sufficient anticholera-immune serum to produce complete agglutination of the bacteria. He then centrifuged and decanted the supernatant fluid and resuspended the sediment in enough distilled water to make a rather thick emulsion. This he added to two test tubes in equal amounts. To tube No. 1, he added distilled water and to tube No. 2, physiological saline. These tubes were then shaken and centrifuged and resuspended as before, i.e., the sediment of tube 1 in distilled water and of tube 2 in saline. The tubes were then shaken and allowed to stand. Bordet says, "It is found that clumps form rapidly in the tube containing salt solution, but that the bacteria remain indefinitely in suspension in the tube containing distilled water." He also removed some of the distilled water emulsion and added a small amount of sodium chloride and obtained rapid agglutination.

Bordet thus concluded that the first phase was a specific adsorption of antibody (agglutinin) by the antigen (bacteria) and that the second phase was one of agglutination in which the sensitized cells were clumped upon the addition of electrolytes. It should be noted that the antibody was not removed by washing in either distilled water or salt solution. For a brief discussion of the physical chemical concepts involved in flocculation, the student is referred to the discussion of colloids included in the appendix.

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EHRLICH'S VIEW.—This two-phase hypothesis of Bordet was vigorously opposed by Ehrlich* who conceived of agglutinin as having chemical groups that caused clumping. Referring to Bordet's work he says, "The attempt has been made to interpret the immunity reactions from the standpoint of colloid chemistry. . . . I see absolutely no advantage in such a method, and I have grave fears that it will result in checking further progress along this line. Structural chemistry, on the other hand, has not only served to explain all the phenomena in immunity studies but has also proved a valuable guide in indicating the lines along which further progress might be made."

This difference of opinion between those holding to the physico-chemical theories and those equally ardent advocates of theories based upon structural chemistry led to an intensive study of antigens and the factors governing specificity and to the mechanism of the immunological reactions. The phenomenon of specificity has been discussed at length in Chapters XVII, XVIII, and XIX.

Subsequent Lines of Research.—In the studies of the mechanism of clumping or agglutination of bacteria by immune serum, three interesting lines of investigation have contributed to our present concepts. These may be enumerated as follows:

1. Studies to determine whether the law of multiple proportions holds when bacteria combine with agglutinin.

2. Studies of the effect of varying salt concentrations on membrane potential and cohesive forces of unsensitized and sensitized particulate matter that has previously been made to adsorb a specific protein and bacteria before and after the adsorption of agglutinin.

3. Studies relative to the nature of cellular surfaces and molecular orientation before and after the adsorption of specific agglutinins. This work has been done largely by Mudd and Mudd (1924, 1926, 1927) using their "interfacial tension technic."

STUDIES ON EFFECT OF DILUTION ON ADSORPTION.—In 1902, Eisenberg and Volk found that if a constant amount of bacteria were mixed with equal amounts of varying dilutions of immune serum, the bacteria removed a greater percentage of the agglutinin content from the higher dilutions. In the lower dilutions where

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the immune serum was more concentrated, they found that while the same amount of bacteria actually adsorbed more agglutinin than from the higher dilutions, yet the amount adsorbed was a smaller percentage of the total amount present than in the latter.

EFFECT OF FRACTIONAL ADDITION OF ANTIGEN.—In 1905, Crow showed that the way bacteria were added to immune serum influenced the amount of agglutinin adsorbed. He found that if the bacteria were added all at once to the immune serum they adsorbed more agglutinin than if added in fractional amounts with appreciable time intervals between. This is similar to the Danysz phenomenon where more toxin is neutralized by a given amount of antitoxin added all at once than when added in fractional amounts with some time intervening. Bordet's experiment of dye adsorption with filter paper illustrates this.

BORDET'S EXPERIMENT EXPLAINING DANYSZ PHENOMENON.—If a dilute solution of a dye is prepared and a definite sized square of filter paper is added, in small pieces at considerable time intervals, the first pieces added will be intensely dyed while the last added may not be colored at all. These results suggest that the adsorption of agglutinin by bacteria is in accordance with the law of adsorption. It appears that the union does not follow any law of simple proportions.

HEIDELBERGER AND KENDALL'S THEORY OF REACTION MECHANISM.—Since it is thought that the mechanism involved in the formation of immune precipitates is identical with the one involved in the agglutination of bacteria by immune serum, the recent theory of Heidelberg and Kendall* (1935, 1937) relative to the mechanism involved in a bacterial precipitin reaction is of interest. In the summary of their paper (1935) they say that "the precipitin reaction between the specific polysaccharide of Type III pneumococcus and homologous antibody found in the horse can be accounted for quantitatively by assuming the chemical combination of the components in a bimolecular reaction, followed by a series of competing bimolecular reactions which depend upon the relative proportions of the components. These reactions would lead to the formation of larger and larger aggregates until precipitation ultimately occurred. The mathematical formulation of this theory on

*Heidelberg and Kendall: *J. Exper. Med.* 61: 563, 1935.

the basis of the mass law is described. The derived expressions are shown to be in accord with the experimental findings and the constants used in these expressions are shown to have definite significance." In subsequent studies Heidelberger and Kendall (1937) have shown that the same mathematical expressions hold for rabbit as for horse precipitins in their reactions with pneumococcus polysaccharides. An excellent summary of their work is given by Chase and Landsteiner (1939).

Reference to later work of Heidelberger and also of Hooker, Eagle and others, bearing upon the controversies over the mechanisms involved in cell sensitization and agglutination respectively, is given in Chapter XI.

EARLY CATAPHORESIS EXPERIMENT.—The second line of investigation which has contributed to our present views involved experiments to determine the action of bacteria suspended in a liquid medium through which a known electric current was passed. Such studies on the cataphoresis of bacteria have been very fruitful. The pioneer work in this field was probably inaugurated by Bechold (1904) and also by Neisser and Friedemann (1904). They noted that bacteria that had adsorbed agglutinin clumped more readily between the electrodes than bacteria that had not adsorbed agglutinin. It suggested to them that perhaps adsorption of agglutinin was associated with a loss of electrical charge by the bacterial cells. In 1905, Pauli explained the agglutination of colloidal particles by electrolytes as due to the neutralization of charges present on the dispersed particles.

POWIS' WORK AND THE CRITICAL POTENTIAL.—The next important contribution was made by Powis (1914) who studied membrane potential changes in oil emulsions and found that coagulation occurred when a critical P.D. of about 30 millivolts was reached.

SIMILARITY TO DENATURED PROTEINS.—In the same year Tulloch suggested that sensitized bacteria, i.e., bacteria that had adsorbed agglutinin, were comparable to denatured proteins.

BUCHANAN'S SUGGESTION (1919).—Buchanan reviewed the whole subject of agglutination in 1919 and suggested that in a bacterial suspension, the repelling forces resided in the similarity of charges on the bacterial cells and these forces are opposed by those of surface tension operating to cause agglutination.

In 1921 Coulter showed that the most favorable pH for the clumping of unsensitized red cells is 4.75 and that this shifts to 5.3 when agglutinin is adsorbed by them. Since this latter is the isoelectric point for serum globulin and since agglutinins like all antibodies are precipitated out with the serum globulins, it suggests that the red cells are filmed or coated with antibody globulin when they adsorb agglutinin as illustrated in Figs. 10 and 11.

LOEB (1923).—As further evidence lending support to the conception that globulin is adsorbed by the red cells is the result of Loeb's (1923) studies on the ability of collodion particles to adsorb protein. He shows after adsorption of a film or partial film of protein the collodion particles acquire the properties of the adsorbed substance. According to Northrop (1928) this phenomenon had been observed first by Meyers and Lottermoser in 1901.

NORTHROP AND DEKRUIF (1922).—Almost simultaneously with the appearance of Coulter's work, Northrop and DeKruif (1922, 1923) published the results of their careful investigation of the mechanism of agglutination. They studied the effect of pH on the specific adsorption of antibody globulin which corresponds to Bordet's phase one and found that it does not seem to affect it materially since the adsorption of antibody globulin occurs throughout a fairly wide range of pH values and even when both elements are similarly charged.

SHIBLEY'S SUMMARY OF NORTHROP AND DEKRUIF'S WORK.—Their final conclusions including the results of their studies on phase two of Bordet are concisely summarized by Shibley* (1926) as follows:

“(1) Agglutination is to be considered in terms of two antagonistic forces; a repelling force, due to like electrical charges, which tends to keep the bacteria apart, and ‘cohesive force,’ which makes for adhesion. In any bacterial suspension, all factors that make the repelling force relatively greater than the cohesive force make for stability; and conversely, all factors that reduce the repelling force or otherwise make the cohesive force relatively greater, lead to flocculation. (2) In the case of unsensitized bacteria, electrolytes in lower concentrations, < 0.01 N, affect primarily the potential, and in higher concentrations, $> 0.01 - 0.1$ N, affect primarily the cohesive force. (3) As long as the

*Shibley: J. Exper. Med. 44: 667, 1926.

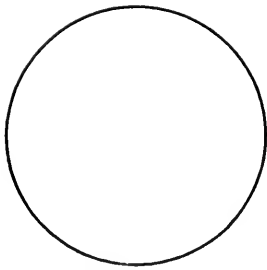


Fig. 10.

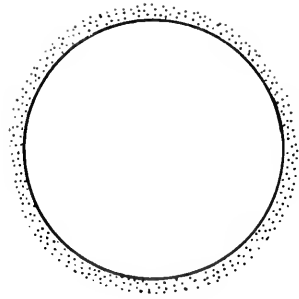


Fig. 11. R. Blood

Fig. 10.—Red cell before adding immune serum containing hemagglutinin.

Fig. 11.—Red cell after adding hemagglutinin partly or completely filmed with antibody globulin absorbed from immune serum.

cohesive force is unaffected, agglutination occurs whenever the charge is reduced by electrolytes to a point below a critical level of about 15 millivolts; that is, the unaffected cohesive force now becomes relatively greater than the force of repulsion. (4) Salt in high concentration depresses the cohesive force of unsensitized bacteria so that no agglutination occurs even though there may be no measurable charge; i.e., the cohesive force is now so small that it is always less than the repelling force. (5) When bacteria are treated with immune sera, their cohesive force is in some manner protected from this depressing effect of strong salt (e.g., physiologic salt solution, etc.) and agglutination is determined solely by the charge; that is, whenever the potential of the sensitized bacteria is reduced by electrolyte to a point below 15 millivolts, the suspension agglutinates."

Shibley continues by saying that "this explains the observation of Bordet, confirmed by Northrop and DeKruif in the course of the work being cited, that electrolytes are essential for specific agglutination. That is to say, the salt, routinely used in the ordinary reactions, reduces the charge on the bacteria so that this charge comes to lie in the 15 millivolt agglutination zone, and the cohesive force of the sensitized bacteria, being insusceptible to the depressing effect of the electrolyte, is now relatively greater than the repelling force and flocculation occurs. (6) Their results refute the idea that combination of antibody and organism is caused by difference of sign of the charges carried by the two substances; but are in agreement with the assumption that the agglutinin forms a film on the surface of the organism."

EFFECT OF SENSITIZATION ON CHARGE.—Shibley then proceeds to offer more experimental evidence in support of the hypothesis that the union of antigen and antibody consists in the coating of the former by the latter. He reports that while both normal and immune sera depress the charge in the pneumococcus, the effect of normal serum is slight when compared with the marked reducing effect of specific immune serum. He studied the effect of specific immune serum for Type I pneumococcus, a strain of a hemolytic streptococcus, *Esch. coli*, *E. typhosa*, *S. paratyphosi* A, *Myco. tuberculosis* and for the Flexner, Shiga and Mt. Desert strains of dysentery bacillus. All of the specific immune sera possessed the charge-reducing effect except the three for dysentery bacilli. The three

immune sera for the latter possessed instead a charge-elevating effect. Since the dysentery bacilli possessed a low charge (3 to 5 millivolts) while the other bacteria mentioned possessed high charges (23 to 40 millivolts), Shibley concluded that the effect of specific immune serum on its respective antigen is to bring the charge to a common potential level (8-14 millivolts). This is due to the fact that while the antigens differ as to charge and chemical constitution, when they unite with antibody they are alike in that they are coated or filmed with antibody globulin. Since the latter becomes denatured when specifically adsorbed, all of the particles of filmed antigen behave as particles of denatured protein.

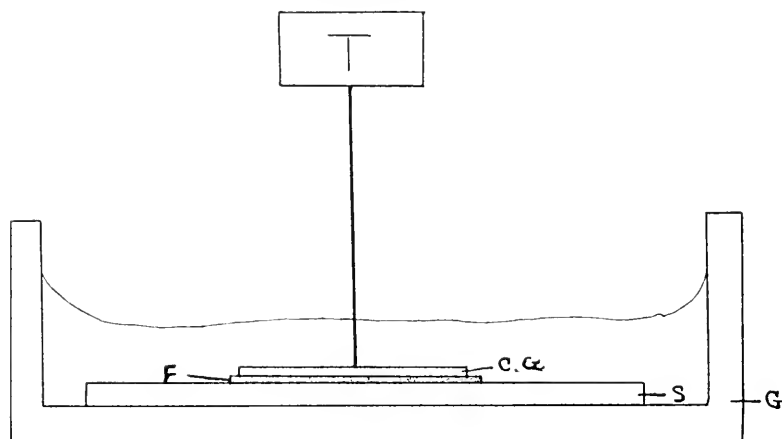


Fig. 12.—Measurement of cohesive force of bacteria.

T, Torsion balance to measure pull necessary to disrupt films (description by Northrop and De Kruif). *C. G.*, Cover glass. *F*, Film of bacteria. *S*, Slide. *G*, Glass container.

Techniques.—MEASURING COHESIVE FORCE.—In order to obtain a rough measure of the cohesive force (stickiness) of bacteria, Northrop and DeKruif introduced an interesting technique. This is described by Northrop as follows: "It occurred to the writer that it might be possible to measure this sticking or cohesive force by determining the force required to separate two films of the suspension. This turned out to be the case. The measurement was made by coating two pieces of glass with a thick smear of the suspension. The glass was warmed slightly in order to cause the particles to adhere to it, and the two films were allowed to rest together in the

solution to be studied. The force required to tear the films apart was then determined by a torsion balance." This is illustrated in Fig. 12.

EXTENT OF SURFACE COATING.—According to Northrop and DeKruif, it is not necessary for the entire surface of the cells to be coated with agglutinin for clumping to occur. They believe that if an eighth of the area is coated, it will suffice. Shibley found that apparently this is more than is necessary provided the other factors are satisfactory.

Studies of Bacterial Surfaces.—Mudd (1924-1927) and Freund (1925) have made extensive studies attempting to determine the nature of the surface of various types of cells before and after adsorption of antibody. The former used the "interfacial tension method" in his investigation and obtained rather significant results. While Mudd and Mudd were primarily interested in the

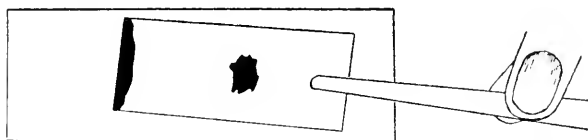


Fig. 13.—Interfacial tension technique of Mudd. (After Mudd and Mudd, J. Exper. Med. 43: 148, 1926.)

phenomenon of opsonification and in phagocytosis, their results definitely support the work of Shibley, Northrop and DeKruif and others in regard to Bordet's phase one where the cells become coated with antibody globulin.

INTERFACIAL TENSION TECHNIQUE.—This "interfacial tension" technique is described by Mudd and Mudd as follows:

"A drop of oil is drawn across a carefully cleaned slide. A small drop of dilute blood cell suspension is drawn along the slide a short distance from and at right angles to the streak of oil. One end of a clean oblong cover slip is touched to the slide and to the oil so that the oil wets the under surface of the cover slip along one end. The other end of the cover slip is now lowered onto the slide, thus spreading the oil into a film under one side of the slip and the blood into a film adjoining it. In the best preparations the blood film does not cover quite all of the area under its end of the slip." This they also illustrate (Fig. 13).

BEHAVIOR OF CELLS.—By means of this technique they investigated first the behavior of red cells before and after they had adsorbed hemagglutinin. Before adsorption they readily passed from the aqueous phase into the oil phase indicating that their surfaces were richly endowed with material readily miscible with oil. Referring to Harkins' work (see Fig. 26) (Appendix, p. 000) this would be interpreted as indicating that the nonpolar groups of the molecules were out.

CHANGE IN SURFACE MOLECULAR ORIENTATION.—After adsorption of agglutinin the surface appeared to contain predominantly polar groups, since the cells were no longer miscible with oil but were readily miscible with the aqueous phase (Ringer's solution). Using the same technique they also studied acid-fast bacteria and observed the same phenomenon. Both Freund and Mudd and Mudd regard the surface of the unsensitized acid-fast bacteria as containing both protein and lipoid. From their work they conclude that the acquisition of polar groups results from adsorbed antibody globulin. This specific adsorption of antibody globulin constitutes the first phase of Bordet's hypothesis.

Antigenic Components and Antibodies.—In Chapters XI and XIX the various possible antigenic components of the bacterial cell were discussed. The student should bear in mind that according to present concepts as many different antibodies may be produced as a result of infection or immunization as there are true antigenic components or hapten-protein combinations.

In the case of motile bacteria there are flagellar (H) as well as somatic (O) antigens. The rough colony type of bacteria contain ϕ antigens (protein) which stimulate the production of corresponding antibodies and the smooth colony types contain carbohydrate-protein complexes that stimulate antibodies that react either with the combination or with the hapten portion in vitro. The latter, however, is frequently not a true antigen by itself.

NONPROTEIN CARRIERS OF HAPTENS.—Recently Zozaya (1932) has referring to Harkins' work (see Fig. 26) (Appendix) this would be interpreted as indicating that the nonpolar groups of the He considers that the physical attachment of the hapten to a colloid makes it an antigen. This revolutionary concept is in line with Zinsser's suggestion that antibody formation is largely a cell surface phenomenon and depends among other things upon

the antigen's being in the colloidal state. In the phenomenon of adsorption of antibodies by the cell which occurs in Bordet's phase one, the student should remember that the immune serum will contain antibodies for each true antigenic constituent of the cell.

Summary

Some of the important points about the mechanism of cellular agglutination by immune serum may be summarized as follows:

1. There are two phases to the reaction as suggested by Bordet.
2. The antibodies are present in the immune serum intimately associated with the globulin fraction.
3. During the first phase the cells specifically adsorb the antibodies corresponding to the antigens present as a surface film composed of antibody globulin. This readily occurs at 0° C. and also at 37° C. to 56° C., and also through a fairly wide range of pH values. It is probably of molecular thickness since it is not detectable by microscopic examination.
4. When the antibody globulin is adsorbed, it seems to become denatured and causes the cells that have adsorbed it to behave as particles of denatured serum globulin with an increase of the cohesive forces under proper conditions.
5. The presence of a proper concentration of sodium chloride leads to a change in membrane potential to within a critical value of 13 to 15 millivolts without a reduction of the cohesive forces.
6. This alteration of membrane potential to within the critical value in combination with the increased cohesive force is responsible for the clumping and sticking together of the cells.
7. Reference is made to the newer concepts of cell sensitization and agglutination offered by Heidelberger and Kendall.

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CHAPTER XIII

PRECIPITINS

Introduction.—Shortly after Gruber and Durham (1896) and Widal (1896) had demonstrated the relative specificity of bacterial agglutinins, Kraus (1897) discovered bacterial precipitins and found them correspondingly specific. He mixed anticholera immune serum with the clear sterile filtrate from a broth culture of the cholera vibrio and incubated the mixture at 37° C. After a short time there appeared a cloudiness followed by flocculation in the tubes. The precipitate settled out, after standing overnight, leaving a clear supernatant fluid. He likewise found that when he mixed a sterile filtrate from a broth culture of *Eberthella typhosa* with antityphoid immune serum a precipitate developed. When antityphoid immune serum was mixed with the filtrate from vibrio cholera or when anticholera serum was mixed with the filtrate from *E. typhosa* no precipitate developed. Thus Kraus showed that the reaction is specific.

Kraus' work was confirmed by Nicolle (1898) and extended by Tchistovitch (1899) and Bordet (1899). The former immunized animals with horse and eel serum and noted the development of specific precipitins in their sera while the latter produced precipitins for cow's milk. The following year Myers (1900) produced precipitins for crystalline egg albumen.

Kraus named the antigen and antibody involved in this type of reaction *precipitinogen* and *precipitin* respectively. This terminology is similar to that used for the antigen and antibody responsible for cellular agglutination where the former is called the agglutinogen and the latter agglutinin (antibody).

According to Uhlenhuth (1909), von Fish became interested in Bordet's observation that precipitins could be produced for cow's milk and extended this by comparing the precipitins for cow's milk and human milk. He concluded that these proteins could be differentiated by the precipitin test. This work was confirmed by Morgenroth (1900) and Wassermann and Schütze. According to Hektoen and Welker (1924), Leblanc (1901), working with

beef serum, found that the euglobulin, pseudoglobulin and albumin fractions could not only be differentiated from each other by the precipitin test but could also be shown to differ from beef hemoglobin.

Schur (1904) says that Wassermann (1900) deserves the credit for first applying the precipitin test to the differentiation of human and animal proteins. Uhlenhuth (1901) is usually credited with the introduction of the test as a medicolegal procedure.

Early Investigations.—**GROUP REACTIONS.**—While Kraus, Wassermann and even Uhlenhuth at first regarded the test as strictly specific Nuttall (1901) early recognized group reactions. Nuttall's observation was immediately confirmed by Uhlenhuth and others and due allowance made for this in the technical procedures worked out by them. Uhlenhuth's conclusion that the test is specific, provided the antigen dilution used is at least 1:1,000, has been repeatedly confirmed and is at the present time an integral part of precipitin technique used in medicolegal cases. Closely related species are differentiated by absorption and precipitin tests.

VALUE IN TRACING BIOLOGICAL RELATIONSHIP.—Shortly after Wassermann's (1900) observation that the blood of different species could be differentiated by the precipitin test, Nuttall (1901, 1902, 1904) investigated the biological relationship within the animal kingdom by means of the precipitin reaction and also recommended that it be used in medicolegal cases. In this work he employed not only the routine qualitative procedure but also a new quantitative technique which he had devised.

It is obvious from a perusal of the early monographs of Nuttall (1904), Kraus (1904), Uhlenhuth (1909) that extensive investigations were conducted relative to precipitins, their formation, nature, specificity, effect of physical and chemical agents upon them, the simultaneous existence of antigen and antibody in sera, the mechanism of the reaction, the importance of precipitins in elucidating various chemical, biological and medical problems, and the source and nature of the precipitates formed in the reaction. This is very evident from the following summary of conclusions which Nuttall (1904) draws from a survey of the literature and from his own experiments.

NUTTALL'S CONCLUSIONS.—He concludes that the rabbit is perhaps the most satisfactory animal for use in obtaining potent immune serum. He used the intraperitoneal method for antigen inoculation and, beginning with a small dose, administered a series of 5 or 6 graded doses, gradually increasing the amounts at three to six day intervals and bled for antibody titration seven to twelve days after the last injection of antigen. He noted a fluctuation in precipitin content during immunization and observed that precipitin persisted in the blood stream in apparently undiminished titer for about one month. (The titer frequently drops much earlier.) He confirmed the observation of Tchistovitch (1899) that precipitins disappear from the blood stream if injections are administered over too long a period. In his opinion antigen and antibody may both be present in the blood without precipitation occurring. (This has since been confirmed by Weil and others and a fairly logical explanation has been offered by Dean.) In regard to measuring the strength of an immune serum, he suggests that the titer may be expressed either in terms of the volume of precipitate formed or "by giving the highest dilution of blood (antigen) with which it reacts, the quantities of interacting substances being stated." In his opinion quantitative determinations are best recorded in terms of the volume of precipitate formed under standard conditions and procedure. He thinks there is evidence indicating the existence of iso-precipitins, auto-precipitins and anti-precipitins being artificially formed in the bodies of treated animals. In regard to normal precipitins he says certain sera may contain them but they are not specific.

He considers that precipitins are antibodies intimately associated with serum globulin. They are more resistant to heat than complement and do not require the latter for their activity. Bacterio-precipitins are inactivated at 58-60° C., while precipitins for animal proteins are inactivated at 68-70° C. As the source of the precipitate he reasons that since the amount formed is frequently out of all proportion to the amount of antigen present, the precipitate very likely comes from the immune serum. Subsequent work has definitely established the truth of Nuttall's assumption. In his conclusions attention is also called to the observation that the presence of heated precipitin serum (precipitoid) interferes with the reaction between fresh, unheated

precipitin and antigen (Mueller, 1902, Eisenberg, 1902, Nuttall 1904). He states that both antigen and antibody resist desiccation but that antibody is, in general, more unstable than antigen. In Nuttall's opinion both antigen and antibody are probably destroyed by tryptic digestion, although he noted that putrefaction of antigen or immune serum does not prevent the formation of a specific precipitate when they are mixed. He found that traces of acids or alkalies reduce the amount of precipitate formed but that, within limits, the concentration of sodium chloride is without effect. He also confirmed Kraus's observation that the precipitate is soluble in an excess of precipitable substance.

In regard to the effect of temperature of incubation upon the rate of the reaction he states that low temperatures retard and high temperatures accelerate it. Temperatures between 5° and 37° C. do not seem to influence the quantity of precipitate formed. He used room temperature incubation in his biological investigation.

Most of these conclusions have been repeatedly confirmed by others and fairly plausible explanations offered for many of the phenomena.

EXTENT OF EARLY USE OF PRECIPITIN TEST.—It is interesting to note that the precipitin test was used quite early for the identification of bacteria, the diagnosis of disease, the study of biological relationships, the identification of human blood and of semen in cases of murder and attempted rape, respectively, and in the detection of food adulteration. It was also used in many immunological studies such as those of Obermayer and Pick (1906), Landsteiner (1903, 1924, 1928) and others on antigenic specificity, the persistence of antigens within the circulation, the mechanism of antigen-antibody reactions as well as many other problems. Subsequent work has dealt largely with the problems raised by these early investigators. Much of it is discussed in the chapters on antigens and specificity. The remainder of this chapter will be devoted largely to a discussion of technique, to the medicolegal aspects of the reaction and some of the factors influencing the formation of precipitates.

Preparation and Prerequisites of a Satisfactory Immune Serum.—For medicolegal work or for use in research it is quite

necessary to have a precipitating serum that is clear, of high potency, and that is specific. Satisfactory sera are best prepared from rabbits or roosters.

INOCULATION OF ANIMALS.—It seems that any of several methods of immunization yields satisfactory results. Since different individuals of the same species vary in their capacities to produce antibody, one should inoculate several rabbits in order to be certain of obtaining a satisfactory antiserum regardless of the particular technique adopted.

INOCULATION OF ANIMALS ACCORDING TO KOLMER.—Kolmer recommends that 0.5 c.c. of the antigen be injected intravenously every day for three weeks and a trial titration made ten days after the last injection. If the titer is low he suggests that 5.0 c.c. be given intraperitoneally and twenty-four hours later another series of intravenous injections be started.

INOCULATION OF ANIMALS ACCORDING TO DEAN.—Dean (1931) recommends that two series of injections be given. Each series consists of six to eight injections, each of 2 c.c. of the antigen at five day intervals. The first few injections are given intravenously, and the remainder intraperitoneally. After a rest of three to six months the second series is given.

METHODS USED IN THIS LABORATORY.—Various methods are used in this laboratory. One is essentially that recommended by Kolmer, another is quite similar to the procedure followed by Nuttall. One or two c.c. of the antigen (serum undiluted, egg white 50 per cent or crystalline egg albumen 1 to 5 per cent) is injected into the marginal ear vein of a rabbit. This is followed three to six days later by either another intravenous injection of the same amount or by an intraperitoneal injection of 4 or 5 c.c. of the antigen. A third, fourth and frequently a fifth injection is given intraperitoneally at 3 to 6 day intervals and the animals bled for antibody titration 7 to 10 days after the last injection. Normal (0.85 per cent) saline is used as a diluent.

Where a rooster is used for precipitin production, one injection of 20 c.c. of whole blood or other antigenic material is given intraperitoneally. After ten or twelve days blood is removed from the heart and titrated for precipitin content. Hektoen recommends

that 1.8 per cent saline be used in preparing dilutions of rooster serum to avoid nonspecific reactions.

WHEN TO BLEED ANIMALS.—In collecting blood from an immunized animal to be used in precipitin work, it is better to bleed the animal just before feeding or after moderate fasting to avoid cloudy or opalescent sera. Undoubtedly much of the difficulty reported in the early literature was due to failure to observe this precaution.

OBTAINING IMMUNE SERUM.—After it is determined that the precipitin content is sufficiently high to react by the ring technique to be described later, with dilutions of antigen of 1:1,000 or higher, the animals are bled to death under aseptic conditions. It is desirable to obtain the serum in a sterile condition, but if contamination occurs the antiserum may be sterilized by filtration, placed in sterile containers, sealed and kept in the refrigerator. Preservatives should not be added, the serum should not be heated or frozen. All cloudy or opalescent sera should be discarded.

TITRATION AFTER UHLENHUTH.—The next step would be the titration of the antiserum. According to Uhlenhuth (1909, p. 815) he prepared three dilutions of antigen 1:1,000, 1:10,000 and 1:20,000 using physiological (0.85 per cent) saline as a diluent. He then placed 4 small, clean test tubes in a rack and with a sterile pipette added one cubic centimeter of sterile saline to tube IV and one cubic centimeter of the 1:20,000 dilution of antigen to tube III, a corresponding amount of the 1:10,000 dilution to tube II and of the 1:1,000 to tube I. He next took a sterile one cubic centimeter pipette graduated to 1/100 of a cubic centimeter and added 1/10 of a cubic centimeter of the clear immune serum to each tube. The tubes were *not* shaken and were incubated at room temperature and observed for five minutes. Tube IV remained clear, but he says that a good serum should give a positive reaction in tube I within two minutes and that within 3 to 5 minutes tubes II and III may become cloudy and a precipitate form and settle out. The titer is recorded as the highest dilution giving a definite reaction.

TECHNIQUE OF NUTTALL.—Nuttall (1904) in his routine qualitative work employed a similar technique. As a rule he used one cubic centimeter of a 1:100 to 1:200 dilution of antigen and to each

tube added 0.05 c.c. of immune serum. The tubes were incubated at room temperature, apparently without shaking, and observed closely during the first five minutes and at intervals for 24 hours. Adequate controls were also included.

QUANTITATIVE TECHNIQUE OF NUTTALL.—In his quantitative technique he employed as a standard, representing 100 per cent precipitate, the amount formed when 0.1 c.c. of antiserum is added to 0.5 c.c. of a 1:100 or 1:200 dilution of homologous antigen. He considered it necessary that the ratio of antiserum to antigen be 20:1 to 200:1 or more. After the antiserum is added the tubes are shaken.

TIME OF INCUBATION.—The tubes are then incubated for twenty-four hours, the supernatant fluid decanted, and the precipitate drawn up into capillary tubes 12 cm. long and having a lumen of 1 mm. The dry end of each capillary tube is sealed and the tubes are allowed to stand vertically for 24, 48 and 72 hours and the volumes (height) are measured.

THE RING TEST.—He says that Ascoli (1903) suggested stratifying immune serum under varying dilutions of antigen and determining the highest dilution of antigen that produces a ring of precipitate at its junction with immune serum. This is essentially the ring technique of Fornet and Müller (1910) and the one which Hektoen (1928) seems to prefer. In this test small clean vials or test tubes having a diameter of approximately 0.5 cm. are placed in a special rack. A series of dilutions of antigen varying from 1:1,000 to 1:10,000 or 1:20,000 are prepared. By means of a capillary pipette a measured amount of saline (0.85 per cent) is put into the last vial, the same amount of the highest dilution of antigen is put into the next vial and so on until vial I receives the same amount of the 1:1,000 dilution of antigen. With another capillary pipette a measured amount of clear immune serum, to be titrated, is stratified in the bottom of the saline control and a corresponding amount stratified under each antigen dilution, beginning with the highest dilution and proceeding to the lowest. Instead of stratifying the immune serum under the antigen dilutions as recommended by Hektoen (1928) some prefer to layer the antigen dilutions over 0.1 c.c. of immune serum that has previously been placed in each vial. The tubes are incubated at room temperature and the titer is recorded as the highest

dilution of antigen that yields a definite ring at the junction with immune serum. Small round-bottomed fermentation vials may be used in this work. A satisfactory serum should react within two to twenty minutes at room temperature.

RANGE OF SPECIFICITY.—As soon as the titer of an immune serum, e.g., antihuman, has been determined, it is necessary to ascertain the range of its specificity. In blood work this is accomplished by setting up a series of titrations, using dilutions of various other bloods, as, for example, Hektoen mentions fish, chicken, rabbit, guinea pig, rat, cat, dog, swine, sheep, beef, horse, goat, monkey and human. In the case of high titered antihuman precipitin serum, one will find that except for the blood of anthropoid apes, cross reactions either will not occur or will be evident in very low dilutions such as 1:10. Ape blood may react in dilutions of 1:100 or 1:200, but not in high dilutions such as 1:1,000, 1:5,000 or 1:10,000 which the homologous human blood gives. When one is working with an anti-beef serum one finds that sheep blood (closely related species) gives reactions in fairly high dilutions; absorption experiments, however, will differentiate clearly between the two. Other closely related species are the horse and mule; dog, wolf and fox; domestic fowl, turkey, goose, duck and pigeon; hare and rabbit.

Practical Use of the Test.—According to Fornet and Müller (1910) and Hektoen (1928) the ring test is satisfactory for all medicolegal work. Dean (1931), however, feels that it is satisfactory for preliminary work but that one should consider the influence of optimal proportions of antigen and antibody upon the reaction in performing the final test.

ADSORPTION AND AGGLUTINATION TECHNIQUE.—In Chapter XI attention is called to a paper by Cannon (1940) in which he describes a more rational method of titrating precipitins. This method is delicate and sharply specific. It promises to be of value for the quantitative study of the relationship of precipitins to various types of hypersensitive reactions.

Optimal Proportion of Immune Serum and Antigen.—In order that the student may better appreciate some of the conditions necessary for the formation of immune precipitates preparatory to a discussion of the Ramon flocculation technique for the titration

of toxins and antitoxins to be discussed in the next chapter, it might be well to consider briefly the question of optimal proportion of reagents and a few other factors.

PROPORTION OF IMMUNE SERUM AND ANTIGEN USED BY NUTTALL.—Nuttall (1901, 1904) realized that certain ratios of immune serum to antigen favor the formation of immune precipitate. In his work he used ratios varying from 20:1 to 200:1 and higher. Danysz (1902) observed the formation of a precipitate when ricin was completely neutralized by antiricin. He found that the ratio of ricin (toxin) and antiricin (antitoxin) that gave the most voluminous precipitate also was the ratio necessary for complete neutralization of toxin by the antitoxin. In 1909 Calmette and Massol obtained similar results with venom and antivenom. They concluded that this offered a reliable method for measuring the antitoxin content of immune serum. While a great many have offered experimental evidence to show that the toxin-antitoxin precipitation is due to bacterial protein and its bacterial precipitin, Bayne-Jones (1928) in an excellent review of the literature as well as his own work concludes that the reaction is between the toxin and antitoxin and not between bacterial protein and bacterial precipitin.

IMPORTANCE OF OPTIMAL PROPORTIONS OF ANTIGEN AND ANTIBODY.—In view of the importance which zoning plays in both the toxin-antitoxin and precipitin reactions it would seem desirable to review Dean's (1931) extensive investigations and also those of others on the influence of optimal proportions of antigen and antibody in the latter reaction. Dean found that when he mixed e.g. a 1:5 dilution of *antihorse* serum with varying dilutions of *antigen* (horse serum), the largest amount of precipitate occurred with a 1:8 dilution of antigen. When he used a 1:10 dilution of antiserum, the largest precipitate occurred in the tube to which he had added a 1:16 dilution of antigen, etc. He obtained similar results with antityphoid serum and typhoid filtrates. He also noted that when either antiserum or antigen was present in relative excess the reaction was delayed. He says that the importance of optimal proportions has been confirmed by Opie (1923), Parker (1923) and Morgan (1923). The latter worked with the soluble specific substance of the pneumococcus and the homologous type of pneumococcus immune serum. More recently

Dean and Webb (1926) have developed a method for the quantitative determination of either antigen or antibody depending upon the optimal ratio of one to the other within the mixture. This is called the *Optimal Proportion Method*.

COARSE TEST OF DEAN AND WEBB.—In this test they first determine the titer roughly by preparing four dilutions of antiserum, i.e., 1:5, 1:10, 1:20, and 1:40 and ten sets of dilutions of antigen ranging from 1:10 to 1:10,000. Dean and Webb (1926) state that the volume of horse serum dilution in each set is 1 c.e., and the quantity of horse serum is halved progressively in each tube of the series. To each tube of set A they add 1 c.e. of undiluted antiserum, to each tube of set B, 1 c.e. of 1 to 5 dilution of antiserum. They include as controls one set of antigen and also tubes containing only antiserum and saline, the two latter to serve as antiserum controls.

OPTIMAL PROPORTIONS FINE TEST.—The object of the experiment is to determine which tube in each set containing varying mixtures of antigen with antibody shows the most rapid formation of a precipitate. In this way they determine the mixture in which the speed of the reaction is greatest. For the experiment cited they found that the optimal ratio of antigen to antiserum was between 1:16 and 1:32. With this information they proceeded to a finer titration. This is described by Dean and Webb (1926) very much as follows: They prepared an initial 1:100 dilution of horse serum (antigen) using a 100 c.e. volumetric flask. Ten tubes were placed in a rack and numbered for convenience 10 to 1 from left to right. A dilution suitable for the test was prepared from the initial 1 in 100 dilution: in this case 1 in 400 was used. Of this 1 in 400 dilution, quantities varying from 1 c.e. to 0.1 c.e. were pipetted into each of the ten tubes. The difference between each tube was therefore 0.00025 c.e. of horse serum. The volume in each tube was made up to 1 c.e. by adding an appropriate volume of saline solution. One cubic centimeter of a 1 in 40 dilution of antiserum was added to every tube. Thus the volume of antiserum in each tube was 0.025 c.e. Controls were also included. The rack containing the tubes in a single line was incubated at room temperature and observed constantly. The progress of precipitation was watched against a dark background properly illuminated by a shielded electric light. The formation of precipitate was observed

with the naked eye and with a reading glass. There appeared at once in tubes 7 to 2 a cloudy opalescence; after five minutes the opalescence was obviously more marked in the center tubes of the rack and tubes 6 and 5 were leading. After twenty-five minutes tubes 6, 5, and 4 were ahead of the others.

TUBE FIVE CONTAINS OPTIMAL PROPORTIONS.—In tube 5 discrete particles formed earlier than in any other tube; within thirty minutes the particles of tube 5 were larger than those of tube 6 or tube 4. Particles were present in all the tubes after forty-five minutes. The largest particles were in tube 5, next largest in tube 4, then in tubes 6 and 3. Tube 5, the first tube to show distinct particles, contained 0.00125 c.c. of horse serum. The proportion of antigen to antibody in this tube was 1 to 20, i.e., as 0.00125 is to 0.025. In general, they found that strong or quickly acting antisera could be used in a dilution of 1 in 40.

UNIT SUGGESTED BY DEAN AND WEBB.—In regard to applying this test to a quantitative estimation of antibody, Dean and Webb (Dean, 1931) reason that it is possible to express the antibody content in units if the ratio figure depends on the antibody content of the serum. To do this they assume that a unit of antibody is contained in the volume of antiserum which forms distinct particles most rapidly with 0.00001 c.c. of normal horse serum. To illustrate the significance of the results, they state that if the optimal antigen-antiserum ratio is found to be 1:200 it indicates that 0.00001 c.c. of horse serum would react with 0.002 c.c. of antiserum (200 times its volume). Therefore 0.002 c.c. of antiserum contains one unit of antibody and one cubic centimeter would contain 500 units of the latter. (Dean, 1931, p. 430.) From this it is obvious that a low antigen-antiserum ratio figure indicates that the antiserum is rich in antibody while a high figure indicates the reverse.

Precipitins Used to Estimate Haptens and Proteins.—Heidelberger (1933) has reviewed the recent literature on precipitins and calls attention to the use of methods, based upon Dean and Webb's optimal proportions procedure, in the estimation of proteins and also specific polysaccharides.

REASON FOR COEXISTENCE OF ANTIGEN AND ANTIBODY IN BLOOD.—Dean (1931) suggests that perhaps the reason that antigen and

antibody, at times, coexist in the blood without the formation of a precipitate is the absence of optimal ratio of concentrations of each.

Nature of Precipitate.—It is generally conceded (Dean, 1931, Zinsser, 1931) that the major portion of the precipitate is made up of serum globulin from the immune serum.

Effect of pH on the Reaction.—The effect of pH on the formation of immune precipitates has been investigated by Hirsch (1923). Using sheep serum and antiserum he found that precipitation occurs over a wide range, i.e., pH 6 to 9.4 with the maximum precipitation occurring near the acid end of the range. Mixtures having an acidity greater than pH 6 showed nonspecific precipitation, while precipitation was inhibited when the reaction was more alkaline than pH 9. Neutralization of the mixtures restored the ability to give specific precipitates.

Effect of Salts on the Reaction.—The effect of varying the concentration of sodium chloride used in the diluent was studied by Nuttall (1904), Dean and Webb (1926), Hektoen (1928), Baier (1933) and others. Dean and Webb found that the optimal proportions of antigen to antiserum did not vary when concentrations of sodium chloride varying from 0.02 to 1.0 per cent were used although the speed of the reaction was affected by the concentration of the salt. In their opinion a concentration of 0.2 per cent NaCl was the most favorable. As the concentration of salt was increased, the reaction was slower. Hektoen recommends that where immune rooster serum is used sodium chloride concentrations of 1.8 per cent be employed to eliminate nonspecific reactions. Recently Downs and Gottlieb (1932) have studied the effects of different electrolytes upon the formation of precipitates. They* found that "certain salts in molar solution inhibit the formation of a precipitate from horse serum and anti-horse rabbit serum, but others do not. All the salts studied inhibit the formation of a precipitate from crystalline egg albumen and its antiserum when present in molar solution." They state that some additional factor besides the valence is of importance in determining precipitation. They found that the thiocyanates showed the maximum inhibitory effect. In regard to the peptizing effect of the thiocyanate, they say, "The thiocyanate ion can there-

*Downs, C. M., and Gottlieb, S.: *J. Infect. Dis.* 51: 460, 1932.

fore be considered to be effective in peptization and ineffective in precipitation of colloids because it does not disturb the aqueous layer stabilizing the hydrophilic particle." Baneroff (1926) expressed the belief, however, that hydration is not sufficient to account for the Hofmeister series, but that the ions act by shifting the equilibrium among the various polymeric forms of water.

Suppression Phenomenon of Landsteiner.—Landsteiner and Van der Scheer* (1924) call attention to a phenomenon previously studied by Landsteiner: i.e., that "A single precipitin will regularly react with other substances if their chemical structure is sufficiently near to that of the homologous antigen." They (1931) have also shown that frequently these chemically similar substances will react with precipitin serum without the formation of a visible precipitate and thus prevent homologous antigen from uniting with the precipitin. This "suppression phenomenon" has been used by many in immunochemical studies and is referred to in later chapters. Heidelberger and Kendall (1933) have suggested that the reason visible precipitates do not form in many instances is due to the solubility of rabbit serum globulin. (See Chapter XIX.)

Haptens.—Attention has been called to the hapten factor in specificity. Type differentiation of red cells is due to the presence of specific chemical substances, nonprotein in nature, coupled with the cellular protein.

Type specificity of pneumococci is due to nonprotein substances (polysaccharides) that are coupled to the pneumococcus protein. In a later chapter, attention is called to the work of Landsteiner and others who created new antigens by coupling various chemical substances to proteins. The type specific polysaccharides of the pneumococcus and the various chemical substances that are coupled either naturally or experimentally to proteins to create specific antigens have been called haptens by Landsteiner. Naturally occurring haptens are very important factors in antigenic specificity as will be seen from a later discussion. It is interesting to note that immune serum will react not only with the protein-hapten combination but also with the hapten fraction alone, with the formation of specific precipitates or the fixation of complement or the suppression of specific reactions.

*Landsteiner and Van der Scheer: *J. Exper. Med.* 40: 91, 1924.

HAPTENS YIELD PRECIPITATES WITH HOMOLOGOUS IMMUNE SERUM.—Antibodies are not produced when the hapten fraction alone is injected but are produced as a rule when the hapten-protein complex is used for immunization. For example, Type II pneumococcus immune serum will agglutinate Type II pneumococci, form a specific precipitate when mixed with a solution of Type II pneumococci or when mixed with a protein-free solution of Type II, type specific polysaccharide. As previously mentioned in this chapter, Heidelberger and Kendall have used the quantitative precipitin test to determine the amount of type specific polysaccharide present in a solution as well as in the standardization of antisera.

REASONS FOR DILUTING IMMUNE SERUM IN AGGLUTININ AND ANTIGEN IN THE OLDER PRECIPITATION TESTS.—It will be observed from the descriptions given in this chapter of various older methods of performing the precipitin test and of using it for quantitative estimations of antigen or antibody that invariably the latter is used in relatively low dilutions, while the antigen is highly diluted. This may seem strange since in previous chapters, where examples of bacterial agglutination are given, it is stated that the suspension of bacteria is kept constant and various dilutions of immune serum are used to ascertain the greatest dilution (titer) giving agglutination.

Zinsser (1930, 1931) gives an excellent discussion of these apparently contradictory procedures and offers an explanation that is held by a large number of immunologists. In the first place it is quite generally believed that serological tests involving agglutination, precipitation or flocculation are essentially alike so far as the mechanisms involved are concerned. While the underlying mechanisms are discussed in the chapters on colloids, agglutination, complement fixation, and flocculation, it would seem desirable to sketch briefly a few commonly held concepts. When bacterial immune serum is added to an homologous suspension of bacteria, it is thought that bacterial cells become to some extent filmed with antibody-globulin and that agglutination results from certain changes in membrane potentials and in cohesive forces, electrolytes being necessary for the reaction. It is found quite practicable to use high dilutions of the immune serum and yet have enough serum globulin per cubic centimeter to film the bacteria. It is thought

that a similar mechanism operates in the precipitin reaction. Here the antigen is a protein in colloidal solution which, because of the smallness of colloidal particles, permits of great surface exposure per unit of mass. The significance of this can best be appreciated by the following example: If a cube of solid material one centimeter on each side, i.e., 1 c.c. in volume and hence having a surface area of 6 sq. cm., is divided into 1,000,000,000,000,000 smaller cubes each being 0.0001 mm. on the side, the total surface would be increased from 6 square centimeters to 6,000 square centimeters. If, however, the original cube is divided into still smaller cubes, each having a side length corresponding to the minimum dimensions of colloidal particles, the total surface exposed would be increased from the original 6 square centimeters to 14.83 aeres. Zinsser (1930) estimates that if a bacterial cell were divided into particles the size of the antigenic particles in colloid solution, the total surface exposed would be increased 10,000 times.

It will thus be seen that in the precipitin reaction the antibody globulin is being filmed upon colloidal particles or aggregates of antigen and because of the colloidal state of the antigen, the surface to be filmed is relatively much greater than in the case of the filming of bacteria for agglutination. For this reason the antigen can be highly diluted, but the antibody globulin can be diluted only moderately if it is to film the antigenic particles adequately.

The method suggested by Cannon et al. reduces the surface of antigen by filming it onto collodion particles and thus permits of a real titration of the antibody. It is a more rational method than the ring technique.

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CHAPTER XIV

TOXINS AND ANTITOXINS

Toxins Defined.—In earlier chapters antigens have been defined as colloids (thought to be invariably proteins or protein-hapten complexes) which when injected into the blood stream, body cavities or tissues of a suitable animal, stimulate the production of specific reacting substances (antibodies). Toxins are antigenic poisons (Coca, 1925). Their antigenic property sets them off from all other poisons since it is well established that neutralizing substances are never produced against cyanide, strychnine, morphine, ptomaines, endotoxins, etc., but only against true toxins. Attention has also been called to the various kinds of toxins found in Nature, all antigenically different from each other. Among these are the phytotoxins (plant toxins), ricin, robin, and abrin; the zootoxins, such as snake venom and scorpion toxin; and finally the bacterial toxins in which the medical student is primarily interested.

TRUE TOXINS.—Only a few bacteria are known to produce antigenic poisons or toxins. The first bacterial toxin discovered was that of *C. diphtheriae*. This important contribution was made by Roux and Yersin (1888, 1889). Two years later Kitasato (1891) discovered tetanus toxin. Since then, true toxins have been described for the following bacteria: *B. pyocyaneus* (Wassermann, 1896); *Cl. botulinum* (Kempner, 1897); *Streptococcus scarlatinae* (Moser, 1902; Gabritschewsky, 1906; Dick and Dick, 1923; Dochez and Sherman, 1924); *Streptococcus erysipclatis* (Birkhaug, 1925); *B. dysenteriae Shiga* (Todd, 1903; Olitsky and Kligler, 1920); *Vibrio septique* (Robertson, 1916); *Cl. welchii* (Bull and Pritchett, 1917) and also toxins of some strains of *Staphylococcus aureus* (Parker, 1924). Perhaps the student can best appreciate the important facts about these various toxins and their respective antitoxins by discussing a few of them separately and also with reference to the particular toxemic disease with which each is associated.

Etiological Agents of Diphtheria and Tetanus Discovered.—In 1883, Klebs recognized what we now know to be *C. diphtheriae*, in sections of a diphtheritic membrane. The following year (1884) Loeffler confirmed Klebs' observation and obtained the organism in pure culture. In studying its virulence for mice, rats, rabbits, guinea pigs, chickens, etc., he found that mice and rats are quite refractory to infection while rabbits and guinea pigs are exceedingly susceptible. He was very much impressed with the fact that when an animal dies following the local injection of the organism the organisms are not, as a rule, generally disseminated throughout the body but remain localized at the point of inoculation. Loeffler concluded that the disease and death of these animals was probably due to some toxic substance produced by the bacteria. In 1888, 1889, Roux and Yersin discovered that sterile filtrates of broth cultures of *C. diphtheriae* are exceedingly poisonous and can produce the classical symptoms, pathological changes and death in susceptible animals.

Within the next two years Kitasato (1889-1891) isolated the tetanus bacillus in pure culture and discovered tetanus toxin. Behring and Kitasato (1890) immunized animals with diphtheria and tetanus toxin respectively and demonstrated specific neutralizing substances (antitoxins) in their blood. In other words, they showed that these toxins are antigenic. They were the first individuals to produce active immunity against a soluble toxin. According to Dean (1913) Behring did the pioneer work in diphtheria antitoxin and collaborated with Kitasato in producing tetanus antitoxin.

Invasive Power of *C. Diphtheriae*.—It is now known that *C. diphtheriae* possesses relatively little invasive power. In a previous chapter attention has been called to recent work on the variation in invasive power of different strains of *C. diphtheriae* (Wells, 1932, Feierabend and Schubert, 1929, Ivanic, Dimitrijević-Speth and Javanovic, 1923). Apparently some strains that are relatively poor toxin producers possess more invasive power than the majority of strains of *C. diphtheriae*. It is thought that this may account for the failure of antitoxin treatment in a few cases treated early where satisfactory results might have been expected.

Effect of Toxin on Lower Animals.—It is interesting to note that the relative immunity to diphtheria toxin which Loeffler observed

in the mouse and the rat is probably due to tissue insusceptibility. They do not possess antitoxin, and no other substance has been found that will explain their refractoriness to diphtheria toxin. According to Coca (1925) toxin circulates when injected into the blood stream of the rat without being either detoxified or combined with the tissues. Coca, Russell, and Baughman (1921) as well as Glenny and Allen (1922) have shown that exceedingly large doses are toxic. It should be remembered that it is impossible to prepare pure toxin and that the toxin injected is present in the filtrate of a broth culture that is at least seven to ten days old. This contains autolysed products of bacteria, by-products of metabolism other than toxin as well as the latter and also various ingredients of culture media. It is to be expected that large amounts of such a complex pharmacological product might profoundly affect many physiological mechanisms that may be involved in the maintenance of a refractory state to one ingredient, toxin.

SYMPTOMS IN SUSCEPTIBLE ANIMALS.—If a susceptible animal such as a rabbit or guinea pig is given a subcutaneous injection of a fatal dose of either a virulent culture or of toxin alone, one observes a fairly definite train of symptoms. There is always an incubation period of several hours which varies with the virulence of the organism or amount of toxin and the size and age of the animal. The significance of the incubation period is unknown. It is generally assumed that this represents the time necessary for the toxin to reach and be bound by the tissues. Richet, according to Zinsser (1931) suggests that the toxin is not poisonous until acted upon by the body. The selective action of toxin has been explained by assuming a difference of chemical affinity for or of solubility in the various tissues of the body.

PATHOLOGY IN LOWER ANIMALS.—After twelve, fifteen, or twenty hours or even several days, where the dose is small, the animals develop an inflammatory edema and necrosis at the point of inoculation. This is destined to show definite extension. Simultaneously with the development of the reaction at the point of inoculation, the animal also shows clinical evidence of illness. It becomes quiet, the coat is roughened, there is some temperature change and weight loss as well as loss of appetite. Death may be made to occur within four or five days if the proper dose

is given. Autopsy shows hyperemia of the kidneys and adrenal glands, numerous small petechial hemorrhages scattered throughout the body and an increased amount of fluid in the serous cavities. The latter represents a serous exudate.

EFFECT ON DOGS, SHEEP, AND BIRDS.—Roux and Yersin (cited by Loeffler, 1913) studied the effect of toxin in sheep, dogs and birds as well as guinea pigs and rabbits. All were found susceptible to its action. They noted that in many cases where the dogs did not die they developed paralysis. Filtrates of 7- to 10-day-old broth cultures produced the same clinical and pathological picture as that produced by the injection of virulent organisms. Dean (1913) refers to an interesting observation of Martin (1898) that an organism may be toxieogenic and yet nonvirulent. This observation indicates that to produce diphtheria the organisms must establish themselves and by the injury of tissues create conditions favorable to the absorption and distribution of toxin.

Diphtheria in Man.—Diphtheria is usually classified as one of the diseases of childhood. Nursing infants rarely contract diphtheria either through lack of exposure or as a result of passive immunity obtained from the mother. Many adults are susceptible.

The incubation period is usually twenty-four to forty-eight hours. While the primary focus of infection may be in a wound or on the mucous membrane of the eye or vagina, it is usually in the nose, throat or trachea. The local inflammatory response to infection is characterized by redness, edema and the outpouring of an inflammatory exudate rich in fibrin which forms a characteristic membrane. This latter, according to Mallory* (1913), "may appear white, dirty white, brownish, grayish or almost black in color." It is made up of fibrin, bacteria, leucocytes, desquamated epithelial cells and cellular debris. The breath of the patient has a peculiar odor which is almost of diagnostic importance. In laryngeal diphtheria the patient is not only injured by the toxin but the membrane found in the trachea may mechanically obstruct breathing and lead to suffocation. There is great variation in the amount of membrane formation in diphtheria. In some cases it may not be observed while in others it is quite extensive and readily noticed.

*Mallory in *The Bacteriology of Diphtheria* by Nuttall and Graham-Smith, Cambridge University Press.

SYMPTOMS.—Clinically, the patient exhibits only a moderate fever, a rapid pulse and more or less prostration. His first complaint may be only that of a sore throat. If a blood count is made, it usually reveals a moderate leucocytosis. In malignant cases there may be extensive enlargement of the regional lymph glands, extreme discomfort and prostration.

PATHOLOGY IN MAN.—Mallory (1913) gives an excellent discussion of the pathology of diphtheria. He says that the local lesions are essentially the same in that they show “degenerative changes in the epithelial cells and underlying tissues combined with an extensive fibrinous exudate from the blood vessels.” Bronchopneumonia, myocarditis and paralysis of the soft palate are not uncommon complications of untreated diphtheria. For further information the student is referred to Mallory’s excellent discussion of autopsy findings on a fairly large series of fatal cases of diphtheria.

CAUSES OF DEATH IN DIPHTHERIA.—Meyer and Gottlieb (1914) consider that diphtheria toxin is a specific vascular poison. The toxin also acts centrally upon the nervous system. Thus the direct injury of heart muscle, vascular system and adrenal glands combined with the effect upon the nervous system (vagus, phrenic, etc.) is thought to explain the profound drop in blood pressure and cardiac death seen in fatal cases.

Source and Nature of Diphtheria Toxin.—The true bacterial “exotoxins” are generally described as secretory products of the bacterial cell. According to this conception they resemble in some respects the extracellular enzymes that are formed and liberated by the cell. Dernby (1926) regards diphtheria toxin as a higher degradation product of proteolysis. Wells (1929) suggests that bacterial toxins may be toxic radicals attached to proteins. According to Eaton (1938) the theory that diphtheria toxin is formed by enzymatic degradation of proteins in the culture media is becoming less tenable in the light of present research. He also says that there is no experimental evidence to support the theory that toxins are conjugated proteins containing a physiologically active prosthetic group. Different strains of *C. diphtheriae* produce qualitatively the same toxin although there are definite quantitative differences even under optimum conditions.

Some strains of *C. diphtheriae* produce toxin of such potency that 0.001 c.c. will kill a 250 gram guinea pig within four or five days, while other strains produce toxins of exceedingly low potency; i.e., the corresponding minimum lethal dose is 0.10 c.c. or more. Maver (1930), working in Jordan's laboratory, carried out an extensive investigation of toxin production of *C. diphtheriae* in synthetic media.* She reports that "the simple mono-amino-acids, such as alanine, phenylalanine, valine and especially glycine, were more effective in stimulating growth in a synthetic medium than the more complex mono-amino-acids that were tried." She found that a modification of Braun and Hofmeier's synthetic medium containing glycine, a fourfold amount of cystine and asparagin or ammonium succinate instead of sodium asparate was quite satisfactory. One strain of *C. diphtheriae* grown in this medium yielded a toxin of which 0.1 c.c. constituted a minimum lethal dose and 0.0001 c.c. a skin test dose.

When broth containing toxin is subjected to dialysis, the toxin dialyzes slowly but relatively more rapidly, according to Wells, than proteins are observed to dialyze. The toxin is precipitated by protein precipitants such as alcohol, ammonium sulphate, etc., which indicates that it is either a protein or closely associated with the proteins. In this connection it is interesting to note that proteolytic enzymes are said to destroy them. Toxins may be preserved for long periods of time if kept at a low temperature and protected from light. Both ultraviolet light and roentgen rays destroy them.

EFFECT OF HEAT, DROUTH, AND CHEMICALS ON TOXINS.—When a solution of diphtheria toxin is boiled for five minutes both its toxic and antigenic properties are destroyed. The toxin is slowly reduced in potency when heated to 45° C. and more rapidly destroyed when exposed to temperatures of 60°, 70° and 80° C., respectively. Dried toxin withstands 100° C. but is destroyed at 150° C. Roux and Yersin (1889) found that small amounts of acid remove the toxic property and that the latter is restored when the acid is neutralized providing the neutralization is effected within a certain ascertained period of time. Wells† (1929)

*Muller, J. H., and Miller, Pauline A., describe a new gelatine-hydrolysate medium that favors the production of diphtheria toxin of high potency, J. Immunol. 40: 21, 1941.

†Wells, H. G.: Chemical Aspects of Immunity, Reinhold Publishing Corporation.

says that in this respect they "behave like peptids in which acids transform cyclic groups to open chains and alkalies restore the cyclic structure."

Eaton (1938) says that toxins may be affected by physical and chemical agents in three ways: (1) they may be denatured or coagulated; (2) their toxic and biologic properties may be reversibly masked by such agents as soaps and lipids; (3) aldehydes, oxidizing agents and halogens may convert the toxin to toxoid.

Toxoid or Anatoxin.—Pick (1908) has given an excellent review of the early literature relative to the effect of physical and chemical agents upon antigens including the toxins. It was recognized quite early that iodine, formaldehyde and certain other chemicals detoxify toxins without destroying their antigenic property. Ehrlich (1903, 1910) noted that when diphtheria toxin is allowed to age its toxic property is diminished although its antigenic property is retained. He named the deteriorated toxin thus produced toxoid. This latter term is now applied to toxin that has been detoxified by formaldehyde or other chemicals that do not destroy its antigenic property. Many unsuccessful attempts were made to prepare a satisfactory diphtheria toxoid for use in immunization before Ramon (1923, 1925) finally succeeded. He suggested that toxin detoxified by adding formaldehyde and incubating for a sufficient length of time at 37° C. be called "anatoxin." Since the term "anatoxin" might be confused with "antitoxin" his suggestion has not been adopted. To avoid any confusion the term "toxoid" is now generally used to signify a detoxified toxin intended for use in immunization. The observation that toxoid is antigenic but nontoxic led Ehrlich to conclude that the toxic and antigenic fractions of the toxin molecule are not identical. His conclusions are borne out by the more recent work on toxoids formed by adding known chemical agents. An excellent discussion of the mechanism of detoxification of diphtheria toxin by formaldehyde is given by Eaton (1937, 1938).

Method of Measuring Toxin and Antitoxin.—Ehrlich (1903, 1910) and his colleagues carried out an extensive investigation

of toxins and antitoxins. He devised methods for the measurement of each and formulated a theory to explain the phenomenon of toxin neutralization by antitoxin. Ehrlich's original method of determining the units of toxin and antitoxin are discussed briefly in Chapter XI.

In 1922 *Ramon* introduced a *flocculation method* for the titration of toxins and antitoxins. Since then, many papers have appeared in which both the mechanism of the reaction and the significance of the results are discussed. Two of the most important publications bearing upon the subject are those of Bayne-Jones (1924, 1928). He says that Danysz (1902) observed the formation of a flocculent precipitate in neutral mixtures of ricin and antiricin. Similar results were obtained by Calmette and Massol (1909) with mixtures of venoms and antivenoms. Nicolle, Cesari and Debains (1920) stratified antitoxin over toxin-gelatin mixtures and noted the formation of a precipitate in certain tubes. Ramon (1922) added a uniform amount of toxin and diminishing amounts of antitoxin to a series of tubes and noted that a flocculent precipitate formed *first* in the tube containing equivalent proportions of toxin and antitoxin. This tube he called the "indicator tube." If the strength of the antitoxin, i.e., units per cubic centimeter, is known, one can readily calculate the amount of toxin per unit of antitoxin in the indicator tube. The amount of toxin which brings about the most rapid formation of a flocculent precipitate when mixed with one unit of antitoxin is called by Ramon the L_f or flocculating dose of toxin. A flocculent precipitate occurs later in other tubes containing either an excess or an insufficient amount of antitoxin but the tube in which the flocculent precipitate *first* forms contains a balanced mixture of toxin and antitoxin. In the following protocol illustrating the Ramon flocculation technique it will be observed that 3.0 c.c. of toxin are added to each of 6 small test tubes. Antitoxin having a strength of 300 units per cubic centimeter was added in amounts varying from 0.18 to 0.06 c.c. The contents of the tubes were mixed and the tubes were placed in a water-bath having a temperature of 54° C. In this particular experiment they were observed at five-minute intervals for one hour. The results are tabulated in Table VII.

TABLE VII

STANDARDIZATION OF TOXIN

Diphtheria toxin—strength to be determined.
 Diphtheria antitoxin contains 300 units per c.c.
 Temperature of bath—54° C.

	TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5	TUBE 6
TOXIN	3.0 c.c.	3.0 c.c.	3.0 c.c.	3.0 c.c.	3.0 c.c.	3.0 c.c.
ANTITOXIN	0.18	0.15	0.12	0.10	0.08	0.06
Time:						
5 min.	Sl. C.	Sl. C.	Sl. C.	Sl. C.	--	--
10 min.	C	C	C	C	--	--
15 min.	C	C	C	C	Sl. C.	--
20 min.	C	C	P	C	C	--
25 min.	C	P	F	P	C	--
30 min.	C	F	F	F	C	--
35 min.	C	F	F	F	C	--
40 min.	P	F	F	F	C	--
45 min.	F	F	F	F	C	--
50 min.	F	F	F	F	C	--
55 min.	F	F	F	F	P	--
60 min.	F	F	F	F	F	Sl. C.

Sl. C.—Slight turbidity

C—Cloudy

P—Granular precipitate

F—Flocculent precipitate

I—Indicator tube

--—No change

1 unit of antitoxin = 0.00333 c.c.

1 = tube 3 (Indicator tube)

 $0.12 \div 0.00333 = 36.0$ units of A.T. in tube 3 $3.0 \div 36.00 = 0.0833$ c.c. (L_f dose of toxin)

1.0 c.c. of toxin is equivalent to 12.0 units of antitoxin

L_f DOSE OF TOXIN.—It will be observed from an inspection of the above protocol that a flocculent precipitate appeared first in tube 3 to which had been added 0.12 c.c. of antitoxin. The antitoxin contains 300 units per cubic centimeter, therefore 0.12 c.c. represents 36 units. Since 3.0 c.c. of toxin brings about the most rapid flocculation of 36 units of antitoxin, one unit of the latter will be flocculated by 0.0833 c.c. of toxin. This amount of toxin constitutes an L_f or flocculating dose. If the L_f dose of toxin is known and the strength of antitoxin is unknown, the latter can be determined by a similar experiment. In practice an experiment similar to the above can be used as a trial titration. In the final titration the difference in the antitoxin content of any two adjacent tubes in the series may be reduced to 0.01 or 0.001 c.c., the range being determined by the trial titration.

Ramon (1922) found that purified antitoxin consisting for the most part of pseudoglobulins is devoid of flocculation power when mixed with toxin. He and also Bayne-Jones confirmed the ob-

servation of Renaux (1924) that a nonflocculating antitoxin can be titrated by the flocculation test if it is mixed with a standard flocculating serum. The results obtained indicate the combined neutralizing value of the mixture and from this the strength of the unknown can be calculated. Neither Bayne-Jones (1928) nor Ramon noted the Danysz phenomenon in any of their experiments. The former reports (1928) that the optimum temperature for conducting the test is 45°-55° C. The speed of flocculation increases with the temperature up to 55° C. Above this temperature the results are irregular. At 0° C. to 5° C. the speed of precipitation is sufficiently slow to permit setting up the test and allowing the tubes to remain in the refrigerator overnight without a flocculent precipitate forming. Definite results are then obtained by incubating at 45° to 50° C.

Eaton (1936) presents an interesting report on the flocculation reaction with purified diphtheria toxin. He offers evidences that the flocculation rate of *purified* toxins is unchanged when the toxins have not been altered by the methods used in purification.

PH OF TOXIN.—The pH of the toxin can vary, according to Bayne-Jones, between 6.8 and 8.4 without affecting the results of the flocculation test. Toxoid, as well as toxin, readily produces flocculation of antitoxin. The L_f dose of toxin represents its antigenic or combining power rather than its toxic strength. In the case of a fresh toxin containing practically no toxoid the L_f dose will equal the L_o dose of toxin. As the L_o dose of toxin is converted gradually to toxoid, the toxicity for guinea pigs diminishes but its flocculation value remains the same. This is true even when it is completely converted to toxoid.

RELIABILITY OF RAMON TEST.—Since the Ehrlich method of titrating toxin determined the toxic rather than the antigenic strength of a toxin, it is evident why the Ramon flocculation rather than the Ehrlich method is used to measure the strength of toxoid which is nontoxic but antigenic. The test is, however, not always a satisfactory index of the antitoxic property of a serum, since the sera of some horses flocculate slowly and yet possess a high antitoxic content. On the other hand, a rapidly flocculating standard antitoxic serum is considered quite reliable in determining the flocculating dose of toxoid.

In regard to the antigens and antibodies involved in the flocculation reaction, there has been much controversy as to whether it is a bacterial precipitation or a toxin-antitoxin flocculation phenomenon. Bayne-Jones (1928) states that he has reviewed and confirmed the conclusion of H. Schmidt (1926) that toxin-antitoxin flocculation occurs independently of bacterial precipitins. Some of the reasons upon which he bases his conclusion may be summarized as follows:

1. The flocculating and agglutinating titers of an antitoxic serum bear no relationship to each other.

2. Bacterial agglutinins may be demonstrated in the supernatant fluid of the indicator tube after flocculation is complete.

3. According to Moloney and Weld (1925) diphtheria bacilli are separable into groups by means of agglutinating serum but all produce a toxin that is flocculated by one antitoxin.

4. When bacterial precipitin is mixed with toxin, the latter is not removed and furthermore the presence of the precipitin does not interfere with the subsequent flocculation of toxin by antitoxin.

5. Various controls also offer further evidence pointing to the specificity of the toxin-antitoxin reaction.

Theories of Toxin-Antitoxin Mechanism.—In regard to the mechanism of diphtheria toxin-antitoxin neutralization, there have been three theories that have received extensive consideration. They were formulated by Ehrlich (1898, 1903, 1910), Arrhenius and Madsen (1907) and Bordet (1909), respectively.

EHRlich.—Ehrlich (1910) says that when he began the study of diphtheria toxin-antitoxin neutralization he regarded the toxin as a simple chemical substance but that his experimental results led him to think of it as a very complex compound of toxic and nontoxic antigenic substances. He concluded that the diphtheria bacillus secretes two antigenic toxic substances: one he called *toxin* and the second, which possesses less affinity for antitoxin, he called *toxone*. To toxin he ascribes the acute symptoms and death, while toxone produces slowly developing emaciation, paralysis and death of the experimental animal. In determining an L_+ dose of toxin, which theoretically would be 101 M.L.D.'s, he found that it is always larger than the theoretical figure, being very frequently 120, 130 or more M.L.D.'s. This led him to believe that in every

diphtheria toxin there develop toxoids with different affinities for antitoxin and that one of these, which he called *protoxoid*, has a greater affinity for antitoxin than has toxin. The fraction of toxoid possessing the same affinity as toxin for antitoxin he called *syntoxoid*. To other toxoids with lesser affinities he gave other names. Thus he built a concept of a toxin spectrum which is extensively discussed in his studies (1910).

He conceived of the union of toxin and antitoxin as resembling the reaction between a strong acid like sulphuric acid and a strong base as, e.g., sodium hydroxide. These react completely in one direction and with only an appreciable degree of reversibility (Wells, 1929).

ARRHENIUS AND MADSEN.—Arrhenius and Madsen (1907) studied the reaction between tetanolyisin and antitetanolyisin and concluded that the reaction between toxin and antitoxin resembled that between a weak acid such as boric and a base such as ammonia. When the latter react an equilibrium is established and there is present a measurable amount of free acid, base and the neutral salt. This theory is apparently accepted by Glenny (1931).

BORDET'S THEORY OF ADSORPTION.—The third theory, that of Bordet, assumes that as antitoxin is added to toxin, there is a partial neutralization of each molecule of toxin by antitoxin instead of the neutralization of progressively large amounts of the toxin until finally all is neutralized by antitoxin. He does not deny the existence of toxoids but considers that Ehrlich's toxone is merely partially neutralized rather than a separate toxin. In his opinion the union of toxin and antitoxin is an adsorption phenomenon in which the mechanism of union is similar to that in other antigen-antibody reactions. Wells (1929) considers that Bordet's theory comes nearer explaining the "zone phenomenon" and the "Danysz effect" than either of the other two theories.

ZONE PHENOMENON.—The "zone phenomenon" is perhaps best illustrated by the trypanocidal effect of immune serum injected into rats infected with trypanosomes. Taliaferro and Johnson (1926), Johnson (1929) and Coventry (1930) find that if one starts with a dose of immune serum that destroys the trypanosomes and injects a series of animals with progressively larger

doses he finds zones of action and of inaction occur. Thus Coventry (1930) states that when she injected rats infected with *T. lewisi* with varying doses of trypanocidal immune serum (expressed in c.c. per 100 gram body weight) she found that 1.1 c.c. had no effect, 1.5 c.c. was markedly trypanocidal, 1.9 c.c. had no effect, 2.3 c.c. only slightly trypanocidal, while 2.7 c.c. was markedly trypanocidal.

OTHER EXAMPLES OF THE ZONE PHENOMENON.—Other examples of what is commonly called the zone phenomenon are frequently observed in antigen-antibody reactions. Larsen and Nigg (1928) observed that in the Wassermann test for syphilis in individuals having both syphilis and leprosy they frequently observed more complement fixation in the tubes containing smaller amounts of patient's serum than in the one containing the maximum amount. When one works with bactericidal sera one notes quite frequently that there are fewer bacteria destroyed in the stronger concentrations of immune serum than in high dilutions. Corresponding examples of the zone phenomenon can also be demonstrated for precipitins and agglutinins. The zone phenomenon is quite characteristic of colloidal reactions.

Danysz Effect.—Another phenomenon which is apparently a colloidal reaction is called the "Danysz effect." Danysz noted that when he added an excess of toxin to its antitoxin the amount of uncombined toxin varied according to the way he added the toxin to the antitoxin. If he added it in fractional amounts with proper time intervals, there was more unneutralized toxin than if the toxin were added to the antitoxin in one operation. A similar phenomenon has been described for other antigen-antibody mixtures. Bordet considers this an adsorption phenomenon and compares it to the adsorption of dye by filter paper. If one tears a piece of blotting paper into very small pieces and adds them one at a time to a solution of dye, they will take up more dye than if the blotting paper is added as one piece (see Wells, 1929, and also Bordet, 1909).

Thus it will be seen that there is considerable evidence indicating that colloidal phenomena play an important part in antigen-antibody reactions. For a more extensive discussion of the physical chemistry of toxins and antitoxins the student is referred to a short report by Maver (1928).

While active immunization against diphtheria in the experimental animal began with the production of antitoxin by Behring, it was not possible to apply it to man until a safe and dependable antigen was produced and some method devised to determine on a large scale the susceptibility of children and adults to diphtheria.

The history of the research which led ultimately to the development of a satisfactory susceptibility test in man is of considerable interest and importance.

In 1909, Römer and Sames introduced the intracutaneous method of titrating toxin and antitoxin. At the present time it is universally employed in determining the virulence of *C. diphtheriae* and is used frequently in the titration of toxin. According to Glenny (1921) it is a convenient guide in following the transformation of toxin to toxoid.

M.R.D.—Römer adopted two units of toxin, the M.R.D. (minimum reacting dose) and the L_r (limit of reaction) dose, respectively. Glenny (1931) has defined the M.R.D. as the least amount of toxin which when injected intracutaneously into a guinea pig will produce, within 36 hours, an area of hyperemia that is at least 5 mm. in diameter. He defines the L_r dose of toxin as the least amount of toxin which when added to one unit of antitoxin will yield a mixture of such toxicity that 0.2 c.c. will produce a minimal skin reaction when injected intracutaneously into a guinea pig. Other doses of toxin such as $L_r/100$ or $L_r/500$ refer to the least amount of toxin which when mixed with 1/100 or 1/500 unit of antitoxin, respectively, will give a minimal skin reaction in a guinea pig. Whether one employs an end point represented by a faint area of hyperemia, or by an area of hyperemia with resulting necrosis, is of importance since they represent different amounts of toxin. Glenny and his associates prefer the former while Römer made use of both end points. Römer (1909) found that 1/500 of an M.L.D. will produce hyperemia only, while 1/250 of an M.L.D. will cause hyperemia followed by necrosis within 72 hours. Glenny, Pope and Waddington (1925), using an improved intracutaneous technique, conclude that 1 M.L.D. is equal to 1,000 M.R.D.'s. Glenny (1931) says that more recent work in which a faint area of hyperemia 4 to 5 cm. in diameter is employed indicates that 1 M.L.D. is equal to 2,000 M.R.D.'s.

Schick Test for Susceptibility.—In 1913 Schick announced that by means of the Römer technique he could determine susceptibility or immunity to diphtheria. He found that susceptible individuals do not possess sufficient circulating antitoxin to neutralize 1/40 or 1/50 of a guinea pig M.L.D. of toxin when it is injected into the outer layers of the skin (intradermally) of the forearm. A positive reaction indicating susceptibility develops within 18 to 26 hours at the site of inoculation and persists for 7 to 15 days. Occasionally delayed reactions are encountered. The positive reaction manifests itself as an area of hyperemia (redness) one centimeter or more in diameter. Also there may be some swelling and induration. As the reaction subsides scaling manifests itself and there remains a brownish pigmentation. A negative reaction indicates that the individual's blood contains at least 1/40 of a unit of antitoxin per cubic centimeter. The test is performed by injecting into the outer layers of the skin a toxin solution of such strength that 0.1 c.c. or 0.2 c.c. contains 1/40 or 1/50 of a guinea pig M.L.D. Since some individuals are hypersensitive to toxin (see Neill, Fleming, Sugg and Gaspari, 1930), it is quite desirable to run controls with toxin heated to 60°-75° C. for thirty minutes. Pseudoreactions commonly disappear within 2 or 3 days (Banzhaf, 1928) and occasionally they are confusing. The time of disappearance and lack of pigmentation are frequently used as criteria in interpreting a reaction as negative rather than the use of a control.

Recently Taylor and Moloney (1939) recommended that the Schick toxin be prepared from "fresh" toxin less than one year old and free from preservatives. They also suggest that the new Schick toxin have twice the toxicity and lower combining power than the standard Canadian dose. They insist that this will increase the percentage of interpretable reactions. Their claims seem to be substantiated by Cameron and Gibbard (1941).

VARIATION IN SUSCEPTIBLES IN URBAN AND RURAL POPULATIONS.—According to data collected by Park (1919, 1933) and others, the number of susceptible individuals in a population varies in different age groups and also with the opportunity afforded by the environment for acquiring immunity from mild contact infection. He found that at birth 15 per cent of the infants born in

the cities are positive as contrasted with 40 per cent of those born in rural communities. Susceptibility in the other age groups he tabulates in Table VIII.

TABLE VIII*

AGE YEARS	PER CENT SCHICK POSITIVE IN CITY	PER CENT SCHICK POSITIVE IN RURAL POPULATION
1- 2	60-70	80
2- 3	60-45	70
3- 6	45-40	60
6-10	40-30	55
10-16	21-16	50
16-30	20-12	45

*Parks and Williams: Pathogenic Microorganisms, Lea & Febiger, publishers.

SUSCEPTIBILITY IN YOUNG ADULTS.—In a series composed of medical students at the University of Kansas and falling within the age groups of nineteen to twenty-three years, there were 28 per cent Schick positive individuals. This represents a mixed group made up of students coming from cities and rural communities. It will be noted from the data in the above table that ages of greatest susceptibility are the second, third and fourth years of childhood.

SUSCEPTIBILITY DETERMINED BY FIRST DOSE OF T.A.T.—In 1923, Park noted that susceptible individuals react to a subcutaneous injection of 1 c.c. of a toxin-antitoxin mixture, having a standard toxicity, with a reaction similar to that obtained by the Schick test. Thus a susceptibility test and the first of three immunizing injections are combined. It is obvious that this has distinct advantages. Since the reaction depends upon the presence of diphtheria toxin, one can readily appreciate why toxoid, which is nontoxic, cannot be used in any susceptibility test, although it is a good immunizing agent. The test suggested by Park is read on the fifth or six day just as is done with a Schick test. The pseudo-reactions are usually gone by the third day. Park says that the highest percentage of pseudoreactions develop in older children and adults. Controls are usually not run with this test.

Active and Passive Immunity.—As mentioned earlier in this chapter, interest in active and passive immunity to diphtheria began with Behring's and Kitasato's discovery that antitoxins can

be produced by immunizing animals with toxin. Behring and others realized that the reason a patient contracts diphtheria is that he lacks circulating antitoxin and that the rational treatment consists in the administration of the latter. It also follows from this observation that susceptible individuals may be temporarily protected from diphtheria by the administration of antitoxin. The presence of circulating antitoxin produced by the body is an example of *active immunity*, while the protection which results when this antitoxin is injected into a susceptible animal is an example of *passive immunity*.

PASSIVE IMMUNITY.—In the *treatment* of diphtheria with antitoxin, there are a number of important facts that must be considered by the physician. He should remember that the disease is due to the effect of toxin upon the tissue cells and that the antitoxin he injects acts only as a neutralizer of toxin and cannot undo any injury to tissue cells that toxin has already produced. It is believed that one large dose of antitoxin will neutralize more toxin than the same amount given in several doses with appreciable intervals of time intervening. These facts warrant the administration of a large dose of antitoxin as early as possible in a case that is clinically diphtheria even before the report on the culture is available and in some cases when the laboratory findings are negative.

Improved methods of purifying diphtheria antitoxin have been reported by Pappenheimer and Robinson (1937), Pope (1938) and Northrop (1941). Northrop says the new antitoxin is about 20 times as active as the original antiserum.

DOSAGE OF ANTITOXIN.—As to what constitutes an adequate initial dose of antitoxin Park and Williams (1933) recommend that for children under fifteen years of age, 3,000 to 5,000 units be given in mild cases, 5,000 to 10,000 in moderate cases, 10,000 to 20,000 in severe cases and 15,000 to 30,000 in malignant cases. For older children and adults the dosage is practically doubled for each type of case.

METHOD OF ADMINISTERING ANTITOXIN.—In regard to the method of administering the antitoxin, they recommend that it be given intramuscularly in mild and moderate cases, both intramuscularly and intravenously in severe cases and intravenously only in malignant cases.

PROPHYLACTIC DOSE OF ANTITOXIN.—When it is desired to protect a susceptible individual who has been exposed to diphtheria, it is customary to administer 500 or 1,000 units of antitoxin subcutaneously or intramuscularly. This will protect for two to four weeks depending upon the rate of elimination from or destruction within the body. Not infrequently Schick tests are made to ascertain whether the contacts are susceptible or immune and only the former given a prophylactic injection of antitoxin.

Active Immunity.—As previously mentioned in this chapter, active immunity to diphtheria toxin was produced first by Behring and Kitasato (1890). Interest in active immunization against toxins resulted immediately in numerous publications. Ehrlich (1892) reported the successful immunization of mice with ricin, abrin and robin. He found that the offspring of immunized female mice possess passive immunity to toxin. This he attributed to antitoxin obtained largely through the mother's milk. Immunity was not transmitted from the father to the offspring. Two years later Ehrlich and Hübener (1894) immunized guinea pigs with tetanus toxin and obtained similar results. Wernicke (1895) treated guinea pigs with toxin and then antitoxin and finally with several injections of toxin. He observed that immune females transmit antitoxin to the offspring but he was apparently in some doubt as to the mechanism involved.

It remained for Anderson (1906) and Theobald Smith (1907) to show definitely that the offspring of an immune female guinea pig owes its passive immunity to antitoxin obtained through the mother's milk. Such immunity persists for about three months.

ACTIVE IMMUNIZATION OF HORSES WITH T.A.T.—In 1897 Park (1903) began immunizing horses with toxin-antitoxin mixtures. It should be remembered that the injection of toxin-antitoxin mixtures formed a part of Ehrlich's method of determining a unit of antitoxin and of standardizing toxins. The first careful and extensive investigation of diphtheria toxin-antitoxin mixtures as antigens was made by Theobald Smith (1909) and Smith and Brown (1910). They found that partially neutralized or even overneutralized mixtures of toxin could be used in producing an active immunity to diphtheria. They suggest that the antitoxin "smuggles" the toxin into the body where it is slowly liberated with resulting antitoxin formation by the tissues of the body.

They also noted marked variation in the degree of immunity which develops in guinea pigs. Active immunity when once established persists throughout the life of the guinea pig. Theobald Smith recommended that the method be used to immunize children to diphtheria.

Behring, who was the first to produce active immunity in the lower animals to diphtheria toxin and to immunize a child passively with antitoxin, was also the first to use toxin-antitoxin mixtures to immunize children against diphtheria. This was in 1913 shortly before Schick published his susceptibility test. Behring's work was interrupted by the war.

SUSCEPTIBILITY TESTS IN NEW YORK.—In 1914 Park, Zingher and Serota determined the susceptibility of a large number of individuals and during the next year immunized about 10,000 infants, children and adults. They were the first to recognize the value of the Schick test in studying the immunizing effect of diphtheria toxin-antitoxin injected into human beings. Park (1932) says that by 1917 they had determined that 80 to 85 per cent of those receiving their 3L₊ preparation at weekly intervals were immune and that in a majority of the cases the immunity lasted several years. In 1918 they started an immunization program which contemplated the immunization of all children in New York City.

PARK'S PIONEER WORK ON ACTIVE IMMUNIZATION.—In their earlier work they noted a small percentage of severe reactions to the 3L₊ toxin-antitoxin mixtures. They also found that the best immunizing mixture is one having such a toxicity that one human dose will kill a 250 gram guinea pig in about four weeks. Mixtures containing less toxin are poor immunizing agents. They accordingly adopted a toxin-antitoxin mixture containing 0.1L₊ dose of toxin per dose (usually 1 c.c.) in place of those containing 3L₊ to 6L₊ doses of toxin.

PREPARATION OF T.A.T.—According to Banzhaf (1928) this is usually prepared in 40 liter lots. Only toxin that has become stable through ageing and with a residual toxin content of at least 5L₊ doses per cubic centimeter is employed. Concentrated and purified antitoxin containing at least 2,000 units per cubic centimeter is selected for the partial neutralization of the toxin. To prepare a 0.1 L₊ toxin-antitoxin mixture, they place 4,000 L₊

doses of toxin in a 2 liter flask and add 3,000 units of diluted antitoxin to the toxin. After mixing, the contents of the flask is then added to enough 0.8 per cent saline containing 0.5 per cent phenol to make the desired volume of 40 liters. The toxin-antitoxin mixture thus prepared is then sterilized by filtration and its toxic property and immunizing value tested by guinea pig inoculation. When a properly balanced mixture is injected into standard weight guinea pigs, one should find that 0.25 c.c. gives no symptoms, 0.5 c.c. may give slight paralysis after 18 days with recovery, 1.0 c.c. paralysis in 14 to 20 days and death with complete paralysis in 20 to 30 days. Five cubic centimeters should kill a standard weight guinea pig in from four to seven days. The pigs that survive are tested five weeks later to determine their resistance to diphtheria toxin. Those that received 0.25 c.c. and 0.50 c.c. of T.A.T. should withstand 5 M.L.D.'s and 10 M.L.D.'s of diphtheria toxin respectively.

ADVANTAGES OF NEW TOXIN-ANTITOXIN MIXTURE.—This new preparation causes fewer local reactions, since it contains less toxin and bacterial protein than the earlier mixtures employed. It is also safer and the first injection will serve for children under eight years of age both as a substitute for the Schick test and to initiate the production of antitoxin. In older individuals the reaction does not correlate so well with the Schick test. This is due to the greater toxicity and larger content of bacterial protein in the toxin-antitoxin mixture to which many older individuals are sensitive and give pseudopositive reactions.

Park (1932) says that since about 1929 they have been using diphtheria antitoxin obtained from goats in the preparation of toxin-antitoxin mixtures. This is done to avoid sensitizing those injected to horse serum.

RESULTS DETERMINED BY SCHICK TEST.—The results of toxin-antitoxin immunization have been determined by means of the Schick reaction and by observing the incidence of diphtheria in school populations and other groups of immunized individuals. In Park's series, the Schick test indicates that only 30 to 40 per cent become immune within 3 weeks after the first injection, approximately 50 per cent at four weeks, 70 to 80 per cent within six weeks and 80 to 85 per cent within 12 weeks.

Harrison (1932) reports the results he obtained by retesting the children in the Washington schools, who had been immunized against diphtheria. He states that 64 per cent were successfully immunized by three injections of toxin-antitoxin. From a review of the literature he concludes "that 70 per cent successful immunizations from 3 injections would be a fair estimate of the general effectiveness of toxin-antitoxin mixture." He suggests that the wide variation in effectiveness reported by various workers is probably due to the use of deteriorated toxin-antitoxin mixtures and the variation in the strength of the toxin used in the Schick test. Owing to the slowness with which immunity develops, it is obvious that toxin-antitoxin cannot be used in the treatment of diphtheria nor to give immediate protection such as is afforded by passive immunization with antitoxin.

Detoxified Toxin.—The early work on the use of detoxified toxin in immunization experiments on the lower animals is referred to by Pick (1908), Madsen (1908) and Loeffler (1913), Dean (1913), Glenny (1931) and Zinsser (1931). They review the literature and give excellent discussions of active immunity to diphtheria produced by both toxin-antitoxin mixtures and detoxified toxins or toxoids. Apparently Behring (1890) and Fränkel (1890) working in Koeh's laboratory succeeded in immunizing animals with toxoid. The former treated toxin with iodine trichloride, while the latter attenuated it by heat (60° C.). According to Rosenau and Anderson (1908), Burehard (1895) and Anderson (1907) showed that formalin would detoxify tetanus toxin. It remained, however, for Glenny and Sudmersin (1921) to determine experimentally for the first time that *diphtheria* toxin, completely detoxified by the action of formaldehyde, retains its antigenic property. According to Park and Schroder (1932) Ramon "adopted the suggestions of Glenny as to the use of formalin, and of Loewenstein of Vienna as to the value of nontoxic toxoid in tetanus" and developed a satisfactory nontoxic diphtheria toxoid. He uses only a toxin of high potency to which he adds sufficient formalin (according to Glenny, 1931) to make a concentration of 0.3 to 0.4 per cent. The formalized broth culture containing toxin and bacteria is incubated for one month at 37° C. It is then sterilized by filtration and its toxicity determined. It should be detoxified to the extent that 6.0 cubic

centimeters produce no symptoms, either local or general, when injected subcutaneously into a 300 gram guinea pig. Banzhaf (1928) recommends filtering the toxin before adding formaldehyde. Moloney and Weld (1925) report that broth cultures of *C. diphtheriae* are more readily detoxified by formalin than the filtered toxin. Larson and Nelson (1924) and Larson, Halvorson, Evans and Green (1925) have detoxified toxin with sodium ricinoleate. They report successful immunization with nontoxic soap-toxin mixtures.

Apparently phenol is not a satisfactory preservative for toxins. Glenny states that any condition such as the shaking of dilutions which favors the local concentration of phenol at the air-liquid surface results in definite reduction in the antigenic value of the toxin solution. Moloney and Weld (1925) report that toxoids obtained by treating phenol-preserved toxin with formaldehyde frequently possess no antigenic value.

Toxoid Specifications of U. S. Public Health Service.—According to Harrison* (1932) all toxoid manufactured or sold in the United States must comply with the specification supplied by the National Institute of Health of the U. S. Public Health Service. He says that these specifications may be roughly stated as follows:

"The toxin before detoxification must contain not less than 400 M.L.D.'s or 5L₊ doses. Detoxification must be so complete that 5 human doses when injected into guinea pigs must show no sign of early or late diphtheria poisoning. The antigenic efficiency must be such that the initial human dose will immunize 80 per cent of guinea pigs in 6 weeks to such a degree that 5 M.L.D.'s of toxin will fail to kill in 10 days." Park (1932) states that a good toxoid should contain at least 8 antigenic or flocculation units per c.e. It is generally agreed that toxoid is a better immunizing agent than toxin-antitoxin. Harrison (1932) recommends that at least 2 doses of 1.0 c.e. each be given with an interval of from three to four weeks. The importance of this interval of time is supported by the experimental work of Glenny and Pope, Waddington and Wallace (1925). When toxoid is diluted, Park states that at least 4 antigenic units should be present in the dose used for immunization. With 3 injections of

*Harrison, W. T.: Am. J. Pub. Health 22: 17, 1932.

such a toxoid he obtained 94 per cent successful immunizations against diphtheria. He says that Volk of Pontiac was successful in immunizing 83.8 per cent with two injections and 47 per cent with one injection of the same toxoid. The objection to the Ramon toxoid is that severe reactions are produced frequently in older children and adults. For this reason Park adopted toxoid for the immunization of pre-school children and toxin-antitoxin for school children and adults. Bigler and Werner (1941) recommend for infants and young children combined immunization against tetanus and diphtheria, using two injections of 1 c.c. or three injections of 0.5 c.c. of the combined toxoids. They prefer intervals of three months or more between injections.

INUNCTION AS METHOD OF IMMUNIZATION.—Park has also tried out immunization with toxoid by inunction using toxoid mixed with lanolin after Loewenstein. Apparently 4 or 5 rubbings made at weekly intervals immunized about 70 per cent of the susceptibles (children) on whom it was used. The only advantages are in those cases in which consent for injection of toxoid cannot be obtained or “in institutions where a nurse can apply it to the children as they enter” (Park and Schroder, 1932).

Alum Toxoids.—Glenny (1931) states that he and his associates reported in 1926 and 1928 that the addition of 0.01 to 0.1 per cent of potassium alum to toxoid increases the antigenic response. Park and Schroder (1932) state that they have had good results although their series is short and the period of observation less than one year at the time of their report. They report guinea pig experiments that yielded interesting results. One large injection of alum toxoid (0.5 c.c.) or two doses of $\frac{1}{8}$ c.c. each at weekly intervals produced immunity in 80 per cent of a series of guinea pigs as compared with 50 per cent and 20 per cent, respectively, for corresponding doses of toxoid without alum administered to a second series of animals.

In 1931 Glenny and Barr recommended that alum toxoid precipitates be used as antigens. They state that from 1 to 2 per cent of alum will precipitate all of the toxoid; the amount of alum required varies with the batch of toxoid. They recommended the resuspension of these precipitates in saline containing 0.5 per cent phenol. They noted that when amounts of alum varying from 3.5 to 10.0 per cent are used, the purity of the precipitate

increases, while the yield decreases. Since alum toxoid is slowly absorbed and remains in the body for a long period of time, while the Ramon toxoid is rapidly eliminated, they believed that it may be possible to immunize with one dose of alum toxoid. Theoretically the latter should be able to supply both the initial and the secondary stimulus, whereas the Ramon toxoid is eliminated before the appearance of antibodies when a secondary stimulus is necessary for maximum antibody production and therefore a second and perhaps a third injection of Ramon toxoid is necessary.

This work of Glenny and Barr has been followed up by Wells, Graham and Havens (1932) and more recently by Graham, Murphree, and Gill (1933). The latter report that "a single injection of from 5 to 10 units of precipitated toxoid has rendered 171 or 92.4 per cent of 185 strongly Schick positive children Schick negative.

"Of 613 children, 592 or 96.6 per cent were Schick negative when tested from two to four months after a single injection. The original immunity status was unknown, but 72 per cent were pre-school children." Ramon (1940) regards the single injection of toxoid as ineffective.

Active-Passive Immunity.—Ramon (1940) has reviewed the work he and his associates have done since 1925 on what they term active-passive immunity to tetanus or diphtheria toxins respectively. Ramon reports that active immunity can be produced by injecting one or, better, two doses of toxoid sometime after the simultaneous injections of the specific antitoxin and toxoid.

They say that this procedure is not only of value in prophylactic immunization but that they are recommending it as a *new treatment of acute tetanus or diphtheria*.

Recommendations of New York City Department of Health.*—

As a result of extensive experience in diphtheria immunization of children, the New York City Board of Health (1940) has made recommendations which may be summarized as follows:

1. Because of the passive immunity acquired from the mother it is unwise to begin immunization of the child before the ninth month of age.

*New York Department of Health, Quarterly Bulletin 8: 2, 1940.

2. Two doses of alum precipitated toxoid produce as high an active immunity as three doses of plain toxoid. Since the fine precipitate is the active immunizing agent a technique should be employed that assures its administration. Because of the occurrence of annoying local reactions following the use of precipitated toxoid the Department of Health is recommending that three doses of plain toxoid be employed instead of the two doses of precipitated toxoid.
3. The Department recommends that the interval between immunizing injections be one month.
4. Experience has shown that a variable proportion of children successfully immunized at the age of nine months "will be found to have very little immunity at the age of four or five years." To bring up the immunity of such children to a protective titer, a single injection of toxoid is sufficient. It is advised that all children immunized in infancy be given a single injection of toxoid shortly before entering school. This is done routinely in the Department's child health stations; the Schiek test is omitted. Children not previously immunized are given the full course of injections.
5. Schiek tests are given after an interval of two or three months following the completion of a course of immunizing injections. To avoid errors in interpretation the sites of injection are examined from the fifth to the seventh day. A positive reaction is indicated by a persistence of redness or a brownish discoloration at the spot.
6. Dosage of plain toxoid (three injections).
 - a. For children ages 6 years or less three injections consisting of 0.5 c.e., 1.0 c.e., 1.0 c.e. respectively.
 - b. For children over 6 years of age three injections consisting of 0.25 c.e., 1.0 c.e., and 1.0 c.e. respectively.

When a reaction occurs, the subsequent injection should be either the same or less depending upon the severity of the reaction. If the Schiek test remains positive after the three injections of toxoid are given, the series of immunizing injections should be repeated. If the Schiek test is still positive the Department feels that further injections are inadvisable.

Summary.—A few of the important points brought out in this chapter as well as some additional information may be summarized as follows:

1. Diphtheria is essentially a toxemic disease. To produce it the organism, *C. diphtheriae*, must be able to establish itself and create favorable conditions for the production and absorption of toxin. While all strains produce qualitatively the same toxin, they differ in the amount they can produce, in their invasive power, and perhaps in their oxygen requirements.

2. Man seems to be the one naturally susceptible host, although the disease can be produced experimentally in guinea pigs, dogs, and many other animals. Mice and rats are refractory to experimental intoxication with diphtheria toxin. Their resistance seems to depend upon tissue insusceptibility rather than antitoxin. Immunity in man is due largely to antitoxin although tissue resistance to invasion and adsorption of toxin may play some part in natural immunity.

3. The units of measurement of toxin are the minimal lethal dose (M.L.D.), minimal reacting dose (M.R.D.), limit of reaction dose (L_r), limes nul or threshold dose (L_o), limes Tod or death dose (L_d), flocculating dose (L_f) and the Schiek skin test dose (1/50 M.L.D.). These are defined and discussed. Ehrlich introduced the units that involve death or protection against death as end points while Römer introduced a method of measuring toxin in terms of the amount necessary to produce a skin reaction. Modifications of his technic have formed the bases of the skin test units such as the M.R.D., L_r and Schiek test dose of toxin. The technique of the Ramon flocculation test and method of determining the L_f dose of toxin and the mechanism of the reaction are also discussed.

4. Diphtheria toxin like all true toxins is an antigenic poison. It is probably secreted by *C. diphtheriae*. According to Eaton it does not appear to be either a cleavage product from the media nor a toxic radical attached to a protein.

When in solution it is relatively thermolabile, being destroyed when heated to 60° C. for thirty minutes. Dried toxin withstands 100° C. but is destroyed at 150° C.

5. Toxin is neutralized but not destroyed by antitoxin. When some batches of toxin-antitoxin mixtures are frozen, they become more toxic (Banzhaf, 1928, p. 749). When a mixture of toxin-antitoxin is injected into the tissues of man or any suitable animal, the toxin is liberated slowly and stimulates the production of antitoxin.

6. Three theories as to the mechanism involved in toxin neutralization by antitoxin are given.

Ehrlich conceived of the reaction as similar to that between a strong acid and a strong base. To explain many clinical and experimental phenomena he postulates the secretion of two toxins, a toxin and a toxone. These differ in their avidity for antitoxin and in the symptoms they produce. In his opinion the toxone causes the late paralysis observed. He also assumes that these toxins readily deteriorate into toxoids that differ in their affinity for antitoxin. His conclusion that the toxic and antigenic properties are not interdependent is borne out by subsequent work.

A second theory is that of Arrhenius and Madsen. They regard the reaction as similar to that between a weak acid and a weak base. This theory is apparently accepted by Glenny (1931) and his associates.

A third theory is called the adsorption theory of Bordet. When antitoxin is added to toxin he believes that instead of neutralizing a fraction of the toxin molecules present, it partly neutralizes every molecule of toxin.

According to this theory Ehrlich's toxone is only a partly neutralized toxin. Bordet explains the zone phenomenon and the "Danysz effect" from the standpoint of physical chemistry. Wells (1929) and others seem to feel that Bordet's theory more nearly explains all of the observed phenomena than any other theory. Ehrlich's original conception that it is analogous to the reaction between a strong acid and a strong base seems to be generally abandoned.

7. The history of the development of passive and active immunity to diphtheria is discussed. Passive immunization is employed in the treatment of clinical diphtheria and to give immediate protection to susceptible individuals who have been ex-

posed recently. In the treatment of diphtheria one large dose should be administered as early in the disease as possible. This is preferable to the administration of the same amount of antitoxin in divided doses. After toxin is bound to tissue, it is difficult to neutralize. The function of antitoxin is to neutralize toxin; it cannot restore injured tissue to normal nor destroy the diphtheria bacillus.

8. It has been ascertained by means of the Schick test that the greatest percentage of susceptibility in a population occurs in the children of preschool age.

Toxin-antitoxin, Ramon toxoid, alum toxoid and alum precipitated and resuspended toxoid are all used in active immunization against diphtheria. At the present time the antitoxin present in toxin-antitoxin mixtures is obtained from goats. This avoids the possibility of sensitizing an individual to horse proteins.

Toxoid is apparently superior to toxin-antitoxin mixtures as an immunizing agent. It has the additional advantage of not containing serum protein of any kind. The Ramon toxoid gives severe reactions in many older children and adults but seems to be nontoxic for children under 8 years of age. Toxoid *cannot* be used to determine susceptibility.

The student should bear in mind that active immunity develops slowly; i.e., within three to six weeks and hence active immunization is of no value in treatment or immediate prophylaxis.

Harrison reports that 64 per cent of the susceptible school children of Washington were successfully immunized with toxin-antitoxin. Park, on the other hand, reports that about 80 per cent are successfully immunized by toxin-antitoxin. In some series he has been even more successful, the percentage reaching 85 or even 90. It will be several years before one can say definitely how much better toxoid and treated toxoid are than toxin-antitoxin. There is considerable evidence indicating that the best immunizing agent is alum toxoid or precipitated toxoid, while the Ramon toxoid is second and toxin-antitoxin third. The quality of the preparation used, the dosage, number of doses, time interval intervening between injections and also between the first injection and the date of retesting are important factors to be considered in evaluating data. Individuals who possess a

small amount of antitoxin are said to develop immunity more rapidly than those who do not have some immunity. In all immunization work one recognizes that the body must be stimulated by the antigen over a relatively long period of time. Where an antigen is rapidly absorbed and perhaps much of it destroyed or eliminated, it is necessary to give several injections, and care must be used as to the time interval between injections. This varies with the physiological capacity of the body to respond. The first injection is called the *primary stimulus*. The subsequent injections are given just far enough apart to keep the antibody content of the blood rising.

9. Ramon's work in active-passive immunity is discussed briefly. Whether or not his new method of treating tetanus or diphtheria will find general acceptance will depend upon a more extensive clinical trial.

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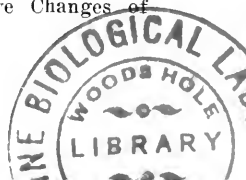
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CHAPTER XV

TOXINS AND ANTITOXINS (CONTINUED)

CONVALESCENT AND IMMUNE SERA

Introduction.—There are a number of diseases other than diphtheria in which the physician may find it desirable to use a specific immune or convalescent serum for diagnosis, prophylaxis or treatment. Among these diseases are scarlet fever and some other streptococcus infections, pneumococcus pneumonia, meningococcus meningitis, measles, poliomyelitis, tetanus, botulism, gas gangrene and tularemia.

It is the purpose of this chapter to bring out a few important immunological facts relative to each of the diseases mentioned.

Scarlet Fever.—Scarlet fever is a toxemic and infectious disease which is moderately contagious. It is quite generally conceded that Dick and Dick (1923) established a beta hemolytic streptococcus as the causal agent. The disease is characterized by fever, leucocytosis, angina (sore throat), a diffuse erythema that can be blanched by scarlet fever antitoxin or by pooled convalescent serum, desquamation, and the development of a lasting immunity following recovery.

The subject of scarlet fever is treated so extensively in a monograph by Dochez (1924) and in all of the standard texts of Pathogenic Bacteriology that only a few salient facts will be mentioned in this chapter.

The evidence which points to the hemolytic streptococcus as the etiological factor may be summarized as follows:

1. Beta hemolytic streptococci are invariably found associated with the disease.

2. Dick and Dick (1923) experimentally reproduced typical cases of scarlet fever in man with pure cultures of these streptococci and apparently satisfied the postulates of Koch.

3. The organisms produce a soluble toxin, when properly cultured, that is capable of producing fever, rash, leucocytosis, desquamation, and antitoxin when injected in sufficient amounts into a susceptible human subject.

4. Specific antitoxin obtained by immunizing horses will blanch the rash of scarlet fever apparently in the same way that pooled convalescent serum blanches it. This is called the Schultz-Charlton blanching test.

5. When streptococcus antitoxin is used in the treatment of severe cases of scarlet fever, there is a striking clinical improvement. Blake and Trask showed that there is a drop in temperature and a rapid clearing up of the rash and other symptoms of toxemia.

6. Susceptibility to the toxin as indicated by the skin test devised by Dick and Dick can be made to disappear by the injection of an adequate number of immunizing doses of toxin.

7. According to the Dicks (1924) immunization with scarlatinal toxin produces immunity to scarlet fever.

8. They also maintain that susceptibility as indicated by the skin test (Dick test) correlates with susceptibility to scarlet fever.

A review of the literature indicates that at the present there is a great deal of confusion and disagreement over the classification and identification of scarlet fever streptococci as well as the interpretation of studies relative to the efficiency of immunization against and the determination of susceptibility to the disease. Our experience with the Dick test (1926) led us to feel that in specific instances it is not as accurate a criterion of susceptibility to scarlet fever as the Schick test is to diphtheria although it may reflect the percentage of susceptibility in a group at large with a reasonable degree of accuracy. To be of maximum value to the physician it must tell him specifically whether a child is or is not susceptible to scarlet fever.

Subsequent investigations of toxins produced by different strains of hemolytic streptococci have revealed wide antigenic variations. Ando and others have shown that the toxic filtrates of streptococcus broth cultures contain two important substances which give rise to red skin reactions when injected intradermally. One of these is the true toxin and the second is a nucleoprotein derivative of the streptococcus. These discoveries explain many but not all of the discrepancies we observed in our studies of the Dick test.

The Dicks report statistics that indicate a very high percentage of efficiency for the susceptibility test.

PROPHYLACTIC IMMUNIZATION.—In regard to the value of prophylactic immunization against scarlet fever, there is much disagreement. The results reported by the Dicks are apparently quite definite. They observed no cases of scarlet fever among 1,191 susceptible interns and nurses who had been immunized with toxin whereas they noted 37 cases of scarlet fever in a control group. Hektoen and Johnson (1934) report statistics from the Durand Hospital which support the findings of the Dicks. There are at present a great many who report results at variance with those just cited. Zinsser, Enders and Fothergill (1939) suggest that active immunization with the Dick toxin may be of value among nurses and interns but do not recommend large scale immunization as a part of a public health program.

THE DICK TEST AND TOXIN VALENCY.—While a negative Dick test indicates a certain amount of immunity to the toxins of scarlet fever streptococci, it is not a measure of immunity to infection with the organisms. It has been shown by Kirkbride and Wheeler (1924) and others that hemolytic streptococci isolated from cases of erysipelas are able to produce scarlet fever toxins. Downs and Stookey (1932) have shown that hemolytic streptococci from cases of puerperal sepsis produce toxins indistinguishable from those produced by scarlet fever streptococci. Similar results have been obtained for hemolytic streptococci from a number of pathological conditions (Wadsworth, 1934). Furthermore, Wheeler (1932), Wadsworth (1934), Trask and Blake (1933), and others have called attention to antigenic differences in the toxins of scarlet fever streptococci. Antitoxin specific for one strain may not neutralize the toxin produced by another.

Since scarlet fever streptococci are of "questionable specificity," Zinsser and Bayne-Jones (1939) recommend that strains that possess as great an antigen valency as possible be selected for toxin production. They call attention to the Dochez N Y 5 strain which produces a toxin of "great antigenic valency." The Dicks have used a polyvalent toxin; i.e., one prepared by mixing the toxins of several strains to meet this requirement.

At the present time it is possible to procure what is regarded as an efficient antistreptococcus antitoxin. It may be used for temporary protection lasting two or three weeks or as a therapeutic agent.

MEASUREMENT OF ANTITOXIN AND TOXIN.—The unit of antitoxin established by the federal government is described as follows: "One unit of antitoxin is the smallest amount of antitoxin which neutralizes 50 skin test doses of scarlatinal streptococcus toxin." (Zinsser and Bayne-Jones, 1939, p. 300.) Wadsworth* states that the skin test dose of toxin is "the least quantity of toxin, which, when injected intracutaneously into persons known to be susceptible to the toxin, will induce a reaction equal to that induced on the same persons at the same time by the injection of a skin test dose of the standard toxin supplied by the U. S. National Institute of Health (Hygienic Laboratory)." This dose is contained in a volume of 0.1 c.c. A positive reaction appears as a pink or red area at least one centimeter in diameter usually within six to twelve hours and begins to fade after about twenty-four hours. It is usually read after twenty-two to twenty-four hours.

SCHULTZ AND CHARLTON BLANCHING TEST.—In 1918 Schultz and Charlton reported that the serum of those convalescing from scarlet fever will blanch the rash of scarlet fever when injected intradermally. Their results have been extensively confirmed. Toomey and Nourse (1924) state that it is necessary to use pooled convalescent sera to obtain an efficiency of approximately 100 per cent in diagnosis. They recommend the intradermal injection of one cubic centimeter of serum and that the test be read not earlier than eight and preferably not until after twenty-four hours. Commercial antitoxin for use in the blanching test is equally satisfactory.

POTENCY OF CONVALESCENT SERUM.—The question arises not infrequently as to the relative value of convalescent serum and commercial antitoxin as a diagnostic prophylactic or therapeutic agent. The interesting work of Rhoads and Gasul (1934) bears directly upon this question. They call attention to the meagerness of reports of the use of convalescent serum for the protection of contacts since its therapeutic value was demonstrated by

*Wadsworth: J. Immunol. 21: 255, 1931.

Weissbecker in 1897. The amount used for protecting contacts has varied. Neff (1922) obtained satisfactory results with doses varying from 15 to 30 c.c., but as a rule smaller doses have been employed. Rhoads and Gasul state that the therapeutic dose has varied from 10 to 240 c.c. and that there is at present a "trend toward smaller doses." They determined the potency of twelve lots of pooled convalescent serum and found that it varied from 250 to 1,000 neutralizing units per cubic centimeter (one unit neutralized one skin test dose of toxin). The government requires that commercial scarlet fever antitoxin have a potency of 15,000 neutralizing units per cubic centimeter. This would indicate that so far as antitoxic potency is concerned the commercial antitoxin is definitely superior to convalescent serum.

PARK'S RECOMMENDATIONS.—Park (1928) calls attention to the value of antistreptococcus antitoxin, convalescent serum and even normal serum or citrated normal blood in the treatment of severe cases of scarlet fever, especially where sepsis is present. For the purpose of treatment with serum or whole blood, he divides scarlet fever cases into two groups.

EARLY MALIGNANT CASES.—In group one he places the early malignant cases seen between the first and the fourth day. In these cases there are observed delirium, restlessness, deep red petechial rash, marked adenitis and severe angina. While specific antitoxin is of great value and may be used, it is also possible to inject intramuscularly convalescent serum or blood taken during the second or third week of convalescence and obtain excellent therapeutic results. The blood is obtained from the median basilic vein of the arm, citrated and injected into various muscles of the patient. Park suggests the triceps, vastus externus (thigh), soleus (calf of leg), and gluteal muscles. In young children he would administer 15 c.c. and in older children 30 c.c. in each of these places. The same sites may be used for subsequent injections since the blood is rapidly absorbed.

About six hours after the administration of the blood, the temperature begins to drop and reaches normal in twenty-four to thirty hours. There occurs also as a rule an early fading of the rash and a disappearance of other evidence of the toxemia.

LATE SEPTIC CASES.—In the second group he places the later septic cases seen between the fifth and the eighth day of the disease.

While the rash may have faded, they have a high, septic temperature (103° to 105° F.), the tonsils and fauces are extensively inflamed and covered with membrane, and there is a persistent cervical adenitis.

This group also responds to antitoxin and convalescent serum or blood. Park states that whole normal blood has been used by Zingher in the treatment of eight cases belonging in this group. He found it to be of definite therapeutic value. Its administration leads to a distinct lowering but not to a striking critical drop in the temperature and to a definite improvement in symptoms.

Immuno-Transfusion.—In addition to the use of antitoxin, normal and convalescent sera in the treatment of scarlet fever with sepsis, there has also been employed the blood of individuals actively immunized with bacterial vaccines. This procedure called *immuno-transfusion* was originally recommended by Wright (1919) for the treatment of typhoid fever. It has not been used extensively.

For a more extensive discussion of scarlet fever streptococci, their toxins and antitoxins, the student is referred to Zinsser and Bayne-Jones (1939) or some other standard textbook of pathogenic bacteriology.

Erysipelas.—Another interesting disease caused by the beta hemolytic streptococci is *erysipelas*. It is an acute infection of the skin appearing as a red, swollen, inflamed area, diffuse at first and later showing a sharp line of demarcation. General febrile changes usually occur. Within a few days the redness and infiltration may disappear and the skin appear normal or perhaps reddish brown, dry and desquamating. The disease lasts usually for one or two weeks. Except in debilitated individuals, the prognosis is usually good.

The bacteria in the lesion are present in the lymph spaces of the corium. As the former advance, the area of inflammation follows. For an excellent discussion of the minute lymphatics of the living skin the student is referred to the work of Hadaek and MacMaster (1933).

TYPES OF LESIONS IN ERYSIPELAS.—A study of the inflammatory exudate in erysipelas shows that it is rich in large mononuclear

phagocytic cells (clasmatocytes). While the inflammation leads to a general systemic reaction, it is not as a rule accompanied by suppuration. Various severe types of the disease may occur. They are, according to Kaufmann, given descriptive names such as erysipelas vesiculosum, erysipelas pustulosum, erysipelas gangrenosum and erysipelas phlegmonosum, respectively. The latter term applies to an inflammation (erysipelas) which becomes diffuse in both the skin and subcutaneous tissues with resulting suppuration. The term indicates the transition to a phlegmon.

STREPTOCOCCI IN ERYSIPELAS.—Fehleisen (1883) not only consistently isolated streptococci from the lesions but also experimentally reproduced the disease in man. His work has been confirmed repeatedly. He concluded that there is a distinct species of streptococcus responsible for the disease and accordingly named it *Streptococcus erysipelatis*. This conclusion has led to a great deal of controversy. Birkhaug and others offer experimental support to the concept of Fehleisen, while Wheeler, Wadsworth, Williams, and others maintain that there is antigenic similarity between hemolytic streptococci from erysipelas, scarlet fever, and other pathological conditions. They are certain that there is no definite and strict correlation between the species of streptococcus and the pathological conditions it produces. Birkhaug and others have studied the soluble toxins produced by hemolytic streptococci from erysipelas and have produced specific antitoxins of definite therapeutic value.

McCaum maintains that scarlet fever antitoxin is equally effective in the treatment of the disease as are the antitoxins produced by Birkhaug and others. At the present time it seems to be settled that a satisfactory antitoxin has been produced and is available in the treatment of erysipelas. To be effective it must be able to neutralize the toxin of the streptococcus involved in the particular case and also supply other specific antibodies. This indicates the importance of using toxins of wide antigenic valences in immunizing animals to produce antistreptococcus antitoxin. The other points at issue are at present unsettled.

Puerperal Sepsis.—Puerperal sepsis or childbed fever may be due to hemolytic streptococci indistinguishable from those found in scarlet fever, or as Harris and Brown* (1929) and others have

*Harris, J. W., and Brown, J. H.: Johns Hopkins Hosp. Bull. 44: 1, 1929.

shown it may be caused by an anaerobic streptococcus first described by Schottmüller in 1910. A review of the literature indicates that puerperal sepsis may be caused by organisms similar to those responsible for "gas gangrene." When the disease is caused by beta hemolytic streptococci, it is conceivable that an antistreptococcus antitoxin is of value if it can opsonize the bacteria and neutralize the toxins of the particular hemolytic streptococcus causing the trouble. In the light of our present knowledge one should not expect it to be of value in the treatment of the disease when the anaerobic streptococcus is the causal agent unless an antigenic relationship exists. Serum treatment of puerperal sepsis has been quite unsatisfactory, very likely for the reasons given above.

Streptococcus Septicemia.—At the present time there is no satisfactory specific serum therapy for streptococcus septicemia. Frequent transfusions are used quite extensively with varying results. Antistreptococcus serums are of apparent value in some cases. It seems that in streptococcus septicemia there is not only a deficiency of antibodies but there also exists a deficient activity and mobilization of the phagocytic cells of the body. Gay has shown that unless the phagocytic army is mobilized and active, the supplying of antibodies will be relatively ineffective. The value of chemotherapy in streptococcal and other bacterial infections is discussed later in this chapter.

IMMUNO-TRANSFUSION IN SEPTICEMIA.—Specific immuno-transfusion is recommended by Brady and Crocker (1932) in the treatment of streptococcus septicemia. Stevenson (1933) reports the use of nonspecific and specific immuno-transfusions in a case of hemolytic streptococcus septicemia, with satisfactory results. For the nonspecific immuno-transfusion normal individuals were given typhoid vaccine intravenously and put to bed for the moderate chill that followed in about one hour. Seven hours later 500 c.c. of a donor's blood was withdrawn, citrated, and injected into the patient with septicemia. This type of treatment was repeated several times with beneficial results. During this period a vaccine was made from streptococci isolated from the patient and a normal volunteer immunized. His blood was used in the final transfusion. There was observed a shift in the Schilling count from left to right after each transfusion.

It is obvious that this method of treatment has a limited application and that its efficiency is probably due to the antibody content which Gay has shown is only one factor in the body's defense against streptococcus infections. The shift in the Schilling count may not be so significant as it would appear since there are many factors to be considered which affect the peripheral leucocyte count.

Blood Banks.—The practice of citrating blood and storing it at 4° to 6° C. is quite common. Kolmer (1940) reviews the literature in connection with his own experimental studies and concludes that blood is "better preserved by the addition of glucose" as suggested by Rons and Turner (1916) or of dextrin as suggested by Maizels and Whittaker (1940).*

Pneumococcus Pneumonia.—Pneumonia, both lobar and broncho- due to the pneumococcus, is one of the principal causes of death in the United States. Until 1929 it was generally accepted that there are three specific types of pneumococci and an heterogeneous group called by Dochez and Gillespie (1913) Type IV. Olmstead (1917) discovered that the latter type is composed of a number of small groups rather than single strains. Recently Cooper et al. (1929, 1932), working in Park's laboratory, have divided Group IV into 29 new types. Additional types have been described by Kauffmann, Mørch and Schmith (1940) and by Walter, Guevin, Beattie, Cotler and Bucca (1941). Their results bring the total number of different pneumococcal types including subtypes to 55. A subtype is one that shows cross reaction with immune serum for another type. Park (1933) said that it is possible to identify fifty or sixty types but only seven or eight are prevalent and therefore important.

TYPES IN LOBAR AND BRONCHOPNEUMONIA.—Sutliff and Finland (1933) report six types as being responsible for 84.1 per cent of their cases of pneumococcus lobar pneumonia. They list them relative to their order of frequency as Types I, II, III, VIII, V, and VII. They report also the order of frequency of the ten types, e.g., III, VIII, XVIII, X, V, VII, XX, II, XI, and XIV which were responsible for 81.1 per cent of their cases of pneumococcus bronchopneumonia. Their findings are quite similar to those of Avery et al. (1917) who pointed out that Types I and II

*See also Studies on Preserved Human Blood, J. A. M. A. 114: 850, 85S, 859, 1940.

were responsible for 66.8 and Type III for 13.0 per cent of 454 cases of pneumococcus lobar pneumonia studied by them.

HISTORY OF SERUM TREATMENT OF PNEUMONIA.—The history of the serum treatment of pneumococcus pneumonia extends back to the beginning of the last decade of the nineteenth century. According to Cole and Dochez (1915), the first to apply pneumococcus immune serum from animals in the treatment of lobar pneumonia were G. and F. Klemperer (1891). They used serum from highly immunized rabbits in the treatment of 18 cases and observed improvement in some and failures in others. Foa and Scabia (1892) and Jansson (1892) also reported some favorable results from the use of immune rabbit serum in lobar pneumonia. Washbourne (1897) and Payne (1897) employed immune sera from horses and donkeys respectively and noted improvement in some of their cases. Eyre and Washbourne (1899) reported that serum sent them by Payne was effective against four strains of pneumococci but ineffective against a fifth which they had. While there were a number of reports favorable to the serum treatment of lobar pneumonia, there were a great many that were definitely unfavorable.

DISCOVERY OF TYPES.—It was not until Neufeld and Händel (1910) and Dochez and Gillespie (1913) had demonstrated definite types of the pneumococcus, and that immune serum is type specific, that any light was thrown upon the failures of serum treatment of lobar pneumonia. Neufeld and Händel called attention to the importance of larger doses of serum in the treatment than had been used previously. They also introduced the mouse protection test as a method of determining the potency of immune serum.

DISCOVERY OF SOLUBLE SPECIFIC SUBSTANCE.—In 1917 two other important contributions to our knowledge of the etiological agent were made. Dochez and Avery discovered the soluble specific substances and as previously mentioned Olmstead reported that Type IV is made up of a number of groups rather than single strains of pneumococci. The soluble specific substances of Types I-XXXII are discussed by Brown* (1939).

CONCENTRATION OF ANTIBODY.—Efforts had been made by Gay as early as 1915 to increase the protective property of specific im-

*Brown, R.: Chemical and Immunological Studies of Pneumococcus Soluble Specific Substances of Types I-XXXII. *J. Immunol.* 37: 445, 1939.

mune serum by methods of concentration, but it was not until 1924 that a satisfactory method was devised. This was accomplished by Felton whose concentrated antibody solutions of Types I and II sera are now used extensively. Efforts to produce an effective Type III antibody solution have been uniformly unsuccessful. Up to the present time the best results obtained in the serum or concentrated antibody treatment of pneumonia are in Type I and to a lesser extent in Type II infections. The introduction of a type specific antipneumococcic rabbit serum promises better results in some types other than I. (Goodner, Horsfall and Dubos, 1937.)

RESULT OF SERUM TREATMENT.—To be effective large intravenous or intramuscular injections should be administered early in the disease; i.e., before the fourth day. Statistics indicate that the mortality *can be reduced* 50 per cent by such treatment. The serum treatment of pneumonia has been most successful with severe cases when combined with chemotherapy. An excellent discussion of serum therapy and chemotherapy in pneumonia is given by Finland, Spring and Lowell (1940). Bullowa (1934) reports definite therapeutic value for Type VIII serum in pneumonias due to the corresponding type of pneumococcus. This is interesting in view of the close relationship of Type VIII and Type III pneumococci and the ineffectiveness of serum therapy in pneumonia due to the latter.

Enzyme Treatment of Type III Pneumococcus Infection.—While serum therapy has been found ineffective in the treatment of Type III pneumococcus pneumonia Dubos and Avery (1931) report that a bacterial enzyme, which they had previously reported (1930) as possessing the property of destroying the capsular polysaccharide of the Type III pneumococcus, is of therapeutic value in the treatment of Type III pneumococcus infection in mice. Goodner, Dubos and Avery (1932) report that dermal infections (Type III pneumococcus) in rabbits undergo an early termination with recovery following the intravenous injection of adequate amounts of the enzyme. The infection is fatal in untreated animals. Whether this method can be used to treat successfully Type III pneumococcus infection in man remains to be determined. It has long been known that the capsule is associated with virulence, and as a result of the extensive work of

Avery, Goebel, Heidelberger and others upon the chemical nature of the type specific polysaccharides, it has been established that they are definitely correlated also with virulence. Apparently when the polysaccharide of Type III pneumococcus is destroyed by the enzyme the organisms are deprived of their most important weapon of offense and can be destroyed rapidly by the body. In the case of serum therapy in pneumococcus pneumonia part of the antibodies injected unite with circulating polysaccharide and thus the amount of the former that is available for opsonification is reduced. In a subsequent paper Dubos (1940) discusses in more detail the enzyme mentioned above and also reports the discovery of a bactericidal, or at least a bacteriostatic, agent (Gramicidin) in extracts of a sporulating soil bacillus. It acts upon gram-positive but not upon gram-negative bacteria and is quite toxic for the animal body.

Chemotherapy in Pneumococcal and Other Bacterial Infections.—It is generally agreed that 1932 marks the beginning of a renaissance in chemotherapy. In that year, Domagk, director of the pathological laboratory in Elberfeld, Germany, discovered that prontosil has marked therapeutic value in streptococcal septicemia in mice. In 1933, Foerster treated successfully a case of staphylococcal septicemia in a ten-month-old infant with prontosil. Domagk (1935) introduced a second therapeutic azo dye which he called prontosil soluble and reported additional success in the treatment of both experimental and clinical streptococcus infections. It remained, however, for Colebrook and Kenny, and Buttle, Gray and Stephenson in England, Love, Bliss and Marshall, and Mellon, Gross and Cooper in America to arouse interest among clinicians and to discover that the sulfanilamide portion of prontosil is the active chemotherapeutic agent. It is interesting to note that sulfanilamide was first prepared by Gelmo in 1908 in the course of his investigations of azo dyes. In 1938 Whitty reported that a new sulfonamide drug, sulfapyridine, synthesized in England by Ewins and Phillips, was of therapeutic value in pneumococcal and staphylococcal infections in mice. In 1939 sulfathiazole and its methyl derivatives were prepared by Fosbinder and Walter, and Lott and Bergeim independently. According to Goodman and Gilman (1941) over 1200 organic compounds containing sulfur have been synthesized and studied. New com-

pounds are being produced and investigated quite rapidly. At the present time sulfathiazole, sulfapyridine, and in certain cases sulfanilamide are being used extensively in the treatment of many types of bacterial infection and as prophylactic chemotherapy in war wounds and a few other conditions. As a rule these drugs are given orally although the sodium salt of sulfanilamide may be injected. It should be emphasized that these drugs are not a cure-all, that toxic reactions may follow their administration, and that their employment requires clinical supervision and laboratory determination of blood levels, etc. Sulfathiazole and sulfapyridine are being employed in conjunction with type specific sera in the treatment of pneumonia. According to Goodman and Gilman (1941), sulfathiazole and sulfapyridine are apparently of value in pneumococcal pneumonia, bacteremia and empyema; in a great variety of staphylococcal, hemolytic streptococcal, meningococcal, and gonococcal infections. According to Hamilton (1941), and Hamilton and Wasson (1941), certain strains of Lancefield's group D streptococci were refractory to sulfathiazole. Major (1940) has reported apparent cures in two cases of streptococcus endocarditis treated early in the disease. None of the sulfanilamide compounds, according to Carey (1940), seem to be of value in rheumatic fever, diphtheria, virus disease, *H. influenzae* or typhoid-dysentery infections. This is interesting since Buttle and associates reported (1937) that sulfanilamide protects mice against *E. typhosa* and *S. paratyphosi*. Kolmer (1940) found sulfanilamide protected mice against *Br. abortus* but not against *Br. suis* and was only moderately protective against *Br. melitensis*. Carey (1940), however, states that sulfanilamide was used in three cases of *Br. abortus* infection without any demonstrable effect.

According to Buttle (1940), the sulfonamide compounds are ineffective against *Cl. oedematiens* but very effective against *Cl. welchii*. For a more extensive discussion of the chemistry, pharmacology, mode of action, indication and contraindication of these compounds, the student is referred to the publications of Mellon, Gross and Cooper (1938), Goodman and Gilman (1941), Lockwood (1940), Carey (1940), and other papers listed in the references at the end of this chapter.

Meningococcus Meningitis.—Meningitis is an inflammation of the meninges or coverings of the brain and spinal cord. While it may be caused by various species of pathogenic bacteria, serum therapy is of value only when the meningococcus is the causal agent. In every case of suspected meningitis, spinal fluid should be obtained and examined as early as possible since, if the meningococcus is found, quarantine can be established and specific polyvalent antimeningococcus immune serum administered as a therapeutic agent. While there are a great many papers appearing relative to meningococcus toxins, there is no definite evidence that the meningococcus produces a true soluble exotoxin. Flexner (1906) developed the serum treatment of meningococcus meningitis. He and others have shown that when serum containing antibodies for the strain of meningococcus present in any particular epidemic is used, the mortality is reduced from 70 per cent in untreated cases to 25 or 30 per cent in those receiving adequate and early treatment with specific serum.

CELLULAR RESPONSE DUE TO SERUM.—Occasionally a physician is called as a consultant in a case of suspected meningitis that has received antimeningococcus serum intraspinally before the spinal fluid was examined. The question arises as to the effect of the injected serum upon the cell count of the spinal fluid. Very little has appeared in the literature bearing upon this subject. Duryea (1930) studied the reaction in two normal individuals to the intraspinal injection of 20 c.c. of concentrated serum preparations. A corresponding amount of spinal fluid was withdrawn before the injections were given. Before treatment the cell count in one volunteer was four and in the other two cells per cubic millimeter. Within one hour the counts were increased to 3,800 and 4,640 per cubic millimeter respectively. At the end of twenty hours the cell count of the first was 180 and of the second 300 per cubic millimeter. Both neutrophiles and mononuclear cells were increased. Such phenomena should be borne in mind in interpreting the laboratory findings in treated cases.

Use of Serum in Virus Diseases.—Convalescent serum is employed frequently in the prevention and treatment of measles and also in the treatment of poliomyelitis. It is very important that serum of any kind intended for such purposes be sterile. A

number of fatal septicemias have resulted from the injection of serum containing virulent organisms. These may result from the presence of virulent organisms in the blood of the donor, accidental contamination may occur through carelessness or in some cases infection may be carried in from the skin of the patient. In a previous chapter attention is called to the importance of testing the donor's blood for syphilis before it is injected into a patient.

Measles.—While there is some controversy over the etiology of measles, it appears to be well established that it is a virus disease. Rake and Shaffer (1940) report culturing the virus in the chorio-allantois of the fertile hen's egg. Park (1928) advises that convalescent serum or plasma be employed prophylactically in such amounts that the child develops a mild form of the disease since this will confer a lasting immunity. If the child is completely protected, it will possess a transient immunity that will last for two to four weeks or as long as sufficient serum remains in the body.

PREPARATION OF CONVALESCENT SERUM.—The method used at the Willard Parker Hospital for obtaining blood is described by Park (1928) somewhat as follows: A sterile 16-gauge Luer needle is inserted into a vein and the blood is collected in a sterile 500 c.c. bottle containing 20 c.c. of a 25 per cent sodium citrate solution and 0.3 gram of oxyquinolin sulphate as a preservative. The cells are allowed to settle and the plasma is removed, tested for sterility, and put up in 3 c.c. vials, 6 c.c. vials, and 30 c.c. bottles. The latter is for institutional use. A sample of uncitrated blood is obtained for the Wassermann test.

PARTIAL RATHER THAN COMPLETE PROTECTION RECOMMENDED.—In 1916 Park and Zingher reported satisfactory results in a short series of cases. They found that 8 c.c. of convalescent serum protected completely, whereas 4 c.c. modified the disease. Since then convalescent serum has been used extensively in New York City with very encouraging results. The great difficulty has been to obtain convalescent serum. Barenberg, Lewis and Messer (1930) compared adult whole blood, convalescent serum and Tunnicliff's immune serum relative to their protective value in measles. They found convalescent serum to be the most effective, although whole blood from adults who have previously had

measles is of great value. It permits of the development of an attenuated form of the disease and is usually available. They found that 6.0 c.c. of convalescent serum completely protected 73 per cent of the children who received it, while the remainder came down with mild attacks. Whole blood from adults who had previously had measles was used in amounts of 30 c.c. Of 56 children so treated after exposure to measles 77 per cent developed a mild form of measles. The Tunnicliff serum was administered but failed to protect or to modify the disease.

IMPORTANCE OF EPIDEMIOLOGICAL FACTORS.—Karelitz and Schick (1935) are of the opinion that epidemiologic factors must be taken into consideration in measles prophylaxis. They review the literature bearing upon this subject and report the results of their studies. They find that in homes where good hygiene is practiced there is a greater percentage of patients showing protection from the administration of convalescent immune serum than in homes where poor hygienic conditions are found.

USE OF POOLED PLACENTAL BLOOD.—Largely to overcome the difficulty of obtaining a serum having protective value in measles, Salazar de Sanza (1932) and Dulitskiy (1932) suggest the use of pooled placental blood or serum. They report quite favorable results from their respective studies of its potency, although they encountered some difficulty in sterilizing it without reducing its therapeutic value. As a result of the experimental work of McKhann and Chu (1933) these objections have been overcome and a concentrated extract, called **immune globulin**, is being prepared for clinical use by at least some of the manufacturers of biologic products.

Polio-myelitis.—It is now quite well established that poliomyelitis is a virus disease. Park (1928) states that from the time that Flexner and Lewis (1910) and also Levaditi and Landsteiner (1910) discovered that serum from convalescent monkeys contains virucidal substances, it has been held that convalescent serum, when administered early, is of prophylactic and therapeutic value. This was also the opinion of Aycock et al. as late as 1929. In 1931 there occurred an extensive epidemic of poliomyelitis in New York. Park carried out an extensive and controlled experimental investigation of the value of convalescent

serum when administered in the preparalytic stage. The treatment did not affect the incidence of paralysis nor the mortality rate. An excellent review of the literature bearing upon the serum treatment of poliomyelitis is given by Harmon (1934). While convalescent serum is not an effective therapeutic agent so far as the prevention of paralysis and death is concerned, it was shown by Flexner to be of definite value when administered prophylactically.

There is no promise, at the present time, that active immunization can be effected by means of a vaccine. This subject is discussed quite extensively by Zinsser, Enders and Fothergill, 1939, p. 767. For those interested in the extensive research relative to the virucidal property of normal, convalescent and immune sera as well as the inactivating effect of anterior pituitary and other substances of endocrine origin, a supplementary list of references is appended. The student is also referred to an excellent brief review of the subject of serum therapy in poliomyelitis given in an editorial (1934). The editor calls attention to the fact that while statistical evidence indicates that serum used therapeutically may not prevent paralysis or death, nevertheless, symptomatic improvement is reported almost universally following the administration of such sera. On the other hand Zinsser, Enders and Fothergill (1939, p. 771) state that it is their opinion that the intraspinal injection of serum may increase the edema of the cord, and therefore be definitely harmful in some cases.

Mumps.—Another virus disease which apparently can be prevented by the injection of specific convalescent serum is epidemic parotitis or mumps. It has an incubation period of about 18 days and is exceedingly contagious. Park (1928) reviews the work of Hess (1915) and Regan (1925) and is inclined to attribute definite prophylactic value to convalescent serum.

Vaccinia and Variola.—There are a few reports in the literature such as those of Blackfan, Peterson and Conroy (1923), Weech (1924) and Mitchell and Ravenel (1924) which indicate that convalescent serum is of value in the prevention of variola. Downs and Stookey (1932, 1933), working in this laboratory investigated the prophylactic and therapeutic value of specific immune serum when administered to rabbits before and after in-

fection with the Armstrong vaccine virus. This virus is uniformly fatal to normal rabbits. When administered before or at the time of infection, the animals are protected, but the serum is ineffective after the virus is once established within the tissues of the body.

Tetanus.—Tetanus is a disease of great antiquity. The causal relationship of *Cl. tetani* to the disease was established by Kitasato in 1889. Specific antitoxins were produced by Behring and Kitasato in 1890. The organisms are present in the intestinal contents of most warm-blooded animals, including man, and hence have a wide distribution in nature. They possess little or no invasive power and gain entrance to the tissues through mechanical injury. Once within the tissues they cannot establish themselves unless anaerobic conditions prevail. It is generally agreed that they remain localized at the point of entry, multiply to a certain extent, and secrete two soluble toxins; one is hemolytic and the other brings about the characteristic symptoms of tetanus. While there are several serological types, all tetanus bacilli produce the same toxin.

It is generally accepted that local tetanus and general tetanus are both due to the action of the toxin on nerve cells in the cord and brain. As to how the toxin reaches the cord and brain there are three variants of a neural transport theory. According to one, the toxin is transported in the nerve fibrils or axones of the motor nerves by some process of "protoplasmic streaming." A second explanation that is widely accepted assumes that the toxin reaches the central nervous system along the endo- or perineural lymphatic vessels of the motor nerves. According to a third theory, the toxin is conveyed to the central nervous system in the tissue spaces of the nerves.

REASON FOR INEFFECTIVENESS OF TETANUS ANTITOXIN IN TREATMENT.—In previous chapters the units of tetanus toxin and antitoxin are given. Evidence is also offered which shows that tetanus antitoxin is of definite value in preventing the disease. In the treatment of general tetanus, large doses of antitoxin are administered. While the antibodies neutralize the circulating toxin, it appears from statistical studies that such treatment does not materially affect the outcome of the disease. When symptoms of

general tetanus appear, it is evident that toxin has already combined with nerve cells of the central nervous system. Wassermann and Takaki discovered that brain tissue forms a very firm combination with toxin, which fact may account for the ineffectiveness of the serum treatment of general tetanus.

In evaluating statistical studies relative to the efficiency of serum and other treatments of tetanus, it should be remembered that there is a definite relationship between the observed incubation period and the mortality rate of the disease. When the incubation period is short, i.e., four to six days, the mortality is approximately 90 per cent, while it is only 50 per cent when the incubation period is ten days or longer. The longer incubation period permits the body to develop active immunity which is not possible when the incubation period is short. There are perhaps a number of other factors that affect the outcome of the disease.

In the preceding chapter attention is called to a new method of treating acute tetanus reported by Ramon (1940). He has called it combined (active-passive) treatment. A more extensive clinical application will determine its value.

Those interested in the methods of preparation of commercial antitoxin are referred to an excellent discussion of the subject in a paper by McCoy (1928) and to the more recent publications of Predtechensky (1931) and of Sneath (1934). An interesting discussion of the present status of tetanus is contained in a paper by Miller and Rogers (1935). Reference to improved methods of antitoxin production are mentioned elsewhere in this chapter and in a preceding chapter.

Gas Gangrene.—There are five pathogenic anaerobes, any one alone or any combination of which may gain entrance into a wound and produce either gas and local necrosis or a fulminating gangrene with severe systemic manifestations. In the latter case death may follow within a few hours. Such types of infection are designated as “gas gangrene.” The five anaerobes listed in the order of their frequency of occurrence in such conditions are: *Cl. welchii*, *Cl. septicum*, *Cl. oedematiens* (*B. novyi*), *Cl. fallax*, and *Cl. histolyticum*. It is generally agreed that the first four of these organisms produce soluble toxins, but there is some disagreement in the case of *Cl. histolyticum*. Zinsser and Bayne-Jones

(1939, p. 629) state definitely that it does not, while Robertson (1929), Peterson and Hall (1923), and others report that it produces a true toxin that is very unstable. The disagreement results from a difference in the conception of the properties of soluble toxins. They all agree that whole cultures injected intramuscularly into guinea pigs produce a rapid digestion of the tissues. Robertson and others state that the digestion is due to an extracellular enzyme that is antigenic and for this reason feel that it comes within the proper definition of a true toxin.

INFECTION, FACTORS AFFECTING THE GERMINATION OF SPORES.—

In natural infection where soil containing spores of various pathogenic anaerobes, along with spores and vegetative forms of other bacteria, gains entrance to a wound, the question arises as to the factors favoring or discouraging the germination of the spores. It is thought that the normal oxygen tension in the tissues is unfavorable to germination. In a wound the presence of dead tissue and a low oxygen tension are probably the most important conditions favoring germination of the spores and the vegetative multiplication of the pathogenic anaerobes. Under these conditions, the bacteria begin to produce toxin quickly, and this leads to the production of more necrosis and better conditions for bacterial growth. Aerobic and facultative anaerobic contaminants present in the wound aid materially in reducing the oxygen tension, and certain chemical tissue debilitants carried in with the infectious agents favor necrosis.

IMPORTANCE OF REMOVAL OF DEVITALIZED TISSUE.—Early in the World War gas gangrene presented a serious medical and surgical problem. It was soon discovered, however, that early surgical removal of all devitalized tissue from a wound and the control of surgical closure by bacteriological examination reduced the incidence of the disease to a very low level. The serum treatment of gas gangrene did not receive much attention until after Bull and Pritchett (1917) had shown that *Cl. welchii* produces a soluble toxin. Previous to that time it was well established that *Cl. septicum* and *Cl. oedematiens* are capable of producing specific antigenic poisons. For a recent discussion of infection by gas-forming anaerobes the student is referred to a paper by Reeves (1935).

Summary of Important Factors Relative to Gas Gangrene.—Some of the important facts that have been established by clinical and experimental investigations of gas gangrene may be summarized as follows:

1. There are three of these anaerobes, i.e., *Cl. welchii*, *Cl. septicum* and *Cl. oedematiens*, that are regarded as the most important ones in gas gangrene. There is at the present time a polyvalent antitoxin capable of neutralizing all three toxins available commercially.

2. **INCUBATION PERIODS FOR VARIOUS TOXINS.**—When the toxin of either *Cl. welchii* or *Cl. septicum* is injected in sufficient amounts intravenously into rabbits, death occurs within five to fifteen minutes. In other words, there is no incubation period following the intravenous injection of adequate doses of these toxins. On the other hand, the toxin of *Cl. oedematiens* never kills acutely but only after a recognizable incubation period. Zinsser and Bayne-Jones (1939) state that when 0.01 c.c. of a potent toxin of this organism is injected intravenously into a 300 or 400 gram guinea pig, death results within forty-eight hours. When lethal doses of these respective organisms are injected into the thigh muscles of guinea pigs, there is an incubation period of several hours in each case. *Cl. welchii* produces death within twenty-four to sixty hours, *Cl. septicum* within twelve to twenty-four hours, and *Cl. oedematiens* within twenty-four to forty-eight hours. Kettle (1919) states that the absence of a cellular response such as is seen in pyogenic infections is characteristic of the lesions due to these anaerobes. While there is not a collection of phagocytic cells in the muscle tissue, he says there may be a few neutrophils in the subcutaneous and connective tissue.

3. **TETANUS IN WOUNDS.**—Spores of *Cl. tetani* not infrequently gain entrance to wounds in the same material that contains spores of the anaerobes responsible for gas gangrene. It is, therefore, advisable to administer tetanus antitoxin simultaneously with the polyvalent antitoxin for the latter organisms.

4. **TISSUES AFFECTED.**—Gas gangrene was regarded at one time as a disease of muscle tissue only, but it has since been found that the toxins are not specific for muscle but act on other tissues as well. Gas gangrene of the abdominal wall following major operations has been reported by Orr (1934) and others.

5. INVASIVE POWER.—While *Cl. tetanus* has no invasive power and, therefore, remains localized at the point of entry, the organisms causing gas gangrene rapidly invade the tissues and blood stream and may become widely distributed in the body.

6. In experimental gas gangrene the areas of gangrene produced by pure cultures of *Cl. welchii*, *Cl. septicum*, and *Cl. oedematiens* exhibit characteristic differences according to Robertson (1929) that can be recognized grossly and microscopically.

DIFFERENCE IN PATHOLOGICAL PICTURE.—*Cl. welchii* and *Cl. septicum* produce relatively large amounts of gas in the tissues while gangrene caused by *Cl. oedematiens* shows little, if any, crepitation. The edema fluid due to *Cl. welchii* is slightly pink in color, that of *Cl. septicum* deep red, while *Cl. oedematiens* produces an extensive colorless gelatinous edema which, when the infection is very rapid, may become pink. In *Cl. welchii* infection the muscles are friable, soft, and have a sodden digested appearance, and a sour rancid odor is evolved. There is no blackening and no suggestion of putrefaction. In gangrene due to *Cl. septicum* the skin and muscles are not friable nor do they appear digested.

7. EFFECT OF TOXINS ON HEART AND ADRENALS.—The effect of these toxins upon the heart and adrenals has been studied extensively. Acute death in cases of gas gangrene is said by Robertson (1929) to be due to heart failure and the exhaustion of the suprarenal glands. The latter, also, occurs in fatal gangrene due to *Cl. septicum*. Robertson and Dale (1920) analyzed the toxic action of *Cl. septicum* by means of a kymograph. Robertson cites this work and quotes Dale's opinion that the toxin exerts both a pressor action and also a poisoning of the heart muscle. The action of the toxin of *Cl. oedematiens* has not been studied extensively.

8. THERAPEUTIC VALUE OF ANTITOXINS.—To be effective, polyvalent antitoxin should be administered before symptoms have appeared or very early in the disease. As a therapeutic agent it is injected intravenously, intramuscularly, and infiltrated locally. Unless the serum contains antitoxins for each of the anaerobes causing the gangrene, it will be ineffective since the one or more whose toxins are not neutralized will still be active. The effect of sulfanilamide and related compounds upon *Cl. welchii*, *Cl.*

tetani, and other anaerobes is discussed earlier in this chapter. For further information the student is referred to papers listed under References to Chemotherapy.

Botulism.—The interesting point about *Cl. botulinum* is that there are three strains of this anaerobe that produce toxins antigenically unlike. While antitoxins are available commercially for types A and B and usually are administered, they are apparently ineffective in the treatment of the disease.

Tularemia.—In 1934 Foshay reported upon the treatment of tularemia with specific immune serum obtained from goats that had received repeated injections of formalized suspensions of *P. tularensis*. The early promising results obtained with this serum have subsequently been disappointing.

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CHAPTER XVI

SERUM REACTIONS

When one administers an animal serum to a patient there is always the possibility that some form of "serum reaction" will result. The kind, amount, and condition of the serum, the method of its administrations, as well as the medical history, present condition, and inherent capacity of the patient to react, are all factors to be taken into consideration by the physician.

Serum reactions may be divided into (1) immediate reactions which occur within a few minutes or within a few hours after the serum is injected, and (2) delayed reactions (serum disease) which manifests itself only after an incubation period of several days or even weeks.

Immediate Reactions.—Individuals who develop immediate reactions may be classified into several groups as follows:

1. The asthenic child who presents a picture of status lymphaticus. Such individuals cannot withstand even mild shocks from almost any cause and may die following the injection of antitoxin. They are even poorer risks if they present a history of asthma. Park and Williams (1933) state that practically all deaths from antitoxin administrations have fallen in this group. Fortunately the general practitioner rarely encounters cases of this type.

2. Nonspecific and anaphylactoid reaction: Lord and Heffron (1938) report that, in a series of 1,755 cases receiving intravenous injections of serum, 7 per cent developed symptoms of difficult breathing, flushing of the face, cyanosis, abdominal or lumbar pain, rapid and weak pulse and a few other symptoms. The same authors report 17.8 per cent of the series as developing *thermal reactions*. Not only febrile reactions but also severe chills occur quite frequently following the injection of antipneumococcic rabbit serum.

In regard to the chill-producing factors present in serum, Goodner, Horsfall and Dubos (1937) report that they can be removed for the most part by heating the rabbit serum to 58° C. for thirty

minutes and then adsorbing it at 4° C. with sterile washed kaolin for fifteen minutes. For a discussion of the treatment of the thermal reaction the student is referred to the reports of Bullowa (1937) and MacLeod (1939).

The term anaphylactoid has been applied to phenomena in the lower animals characterized by anaphylactic-like symptoms, cardiac dilatation, hemorrhages and thrombosis. Apparently the symptoms and pathological changes are nonspecific in that they do not depend upon a natural or acquired hypersensitiveness to a particular substance. Karsner (1928), and Hanzlick and Karsner (1919-20), who have studied this reaction in the lower animals, noted it more frequently following intravenous than intraperitoneal or subcutaneous injections.

3. Specific reactions: (a) A third group of individuals who may give a severe reaction to the injection of antiserum are those who are *naturally* sensitive to such animal protein, just as others are sensitive to feathers, face powder, or ragweed pollen, and develop hay fever or asthma when the exciting agent is injected or reaches the mucous membrane of the nose or intestine or other "shock organ." These people are said to be "atopically" sensitive. They react to much smaller doses of the serum and also react more violently than those who have been made sensitive by a previous injection of serum. They may react violently to intradermal or subcutaneous injections and they cannot be desensitized quickly by administering small doses of the serum. Vaughan and Piper (1937) have collected thirty-five cases from the literature in which death followed the administration of horse serum. In six of these the serum had been administered intracutaneously. Park and Williams (1933) state that about 1 in 10,000 persons develop severe reactions and alarming symptoms, and about 1 in 50,000 die within a short time after the subcutaneous or intramuscular injection of antitoxin. According to Tuft (1937) the incidence of fatal serum reaction is about 1 in 70,000.

(b) Another group giving immediate specific reactions to serum is made up of individuals who have *acquired* hypersensitiveness to horse proteins through a previous injection of horse serum. This may have been in the form of a previous injection of diphtheria, tetanus, Welchii or scarlet fever antitoxin, or from the injection of normal horse serum antipneumococcus, antistrepto-

coccus or antimeningococcus sera. Mild sensitization may occur from the small amount of horse serum in toxin-antitoxin mixtures used in immunization (Hooker, 1924; Tuft, 1932). To avoid the latter source of sensitization, the manufacturers of T.A.T. are using antitoxin obtained from goats, thus avoiding the inclusion of horse serum in the mixture. Park's experience was quite extensive and extremely valuable. He found that the history of a previous injection of horse serum is not a contraindication to the use of antitoxin properly administered. As previously stated statistical studies show that fatalities, when they occur, follow the first rather than the second injection of horse serum, and thus would come under the first three groups mentioned above.

Since the introduction of antipneumococcus rabbit serum by Goodner, Horsfall and Dubos (1937) there has been a growing interest in acquired hypersensitiveness to rabbit serum. While Horsfall, Goodner, MacLeod and Harris (1937) suggest the use of an intravenous test to detect allergy to rabbit serum, Warner (1939) and also MacLeod (1939) conclude that it should not be tried until the cutaneous and ophthalmic tests are made and found negative.

In the modified intravenous test 0.01 c.c. of a 1:10 dilution in 5 c.c. of saline is used for slow intravenous injection. A positive reaction is indicated if during the period of five minutes after administration there is a drop in the blood pressure of 15 mm. of mercury and an increase in heart rate of 15 beats per minute.

The ophthalmic test consists in putting a drop of undiluted or diluted serum in the eye of the patient. It indicates mucous membrane sensitivity and may be negative when the skin test is positive. Rarely is the reverse true. If the skin tests are negative or weakly positive, undiluted serum may be employed for the ophthalmic test but when the skin test is strongly positive dilutions of 1:10 or 1:100 should be employed according to Tuft (1937).

Brown (1938) and also Walzer (1939) report that rabbit serum is not irritating to the skin and therefore properly performed intracutaneous tests do not yield too many nonspecific reactions. They suggest using 0.01 c.c. of a 1:100 or 1:10 dilution of normal rabbit serum for the intracutaneous test. The ophthalmic test is said to be negative frequently in acquired hypersensitiveness to

animal serum while the cutaneous test may be positive. A positive ophthalmic test suggests natural or atopic hypersensitiveness and is regarded by many as a contraindication to the administration of serum.

Delayed Reactions.—1. **SERUM SICKNESS IN MAN.**—Besides the serum reactions described in the preceding paragraphs there is another phenomenon called serum sickness which may follow the administration of horse, hog, rabbit and perhaps other sera. It is not an immediate reaction but develops usually after several days or within two or three weeks. It is characterized by headache, joint pains, fever and a rash. Edema of the face or other parts of the body may occur. Regional enlargement of the lymph nodes is noted quite commonly. The symptoms may last for periods varying from one day (accelerated type), to one week or longer. Relapses have been observed according to Zinsser, Enders and Fothergill (1939). Coca concludes that serum sickness can be produced in 100 per cent of all individuals if as much as 50 c.c. of normal horse serum is given intravenously. It may appear, however, following the administration of much smaller doses. The student should remember that diphtheria antitoxin represents the pseudoglobulin fraction of horse serum that has been precipitated, washed and concentrated. Serum sickness in man is more readily produced with whole serum than with the pseudoglobulin fraction. Harten and Walzer (1939) state that most workers report that the administration of antistreptococcic and antipneumococcic sera gives a higher incidence and greater severity of serum reactions than diphtheria or tetanus antitoxin.

It would appear from the reports of Bradshaw (1939) and Hutchinson (1939), that the incidence of serum sickness is greatly reduced if antitoxin is employed that has been subjected to a special method of treatment involving peptic digestion. Several of those who have perfected enzymatic methods for the treatment of immune serum are Parfentjev (1936), and Coghill, Fell, Creighton and Brown (1940) in America, Pope (1939) in England, and Grabar (1938) and Hanson (1938) in France.

Parfentjev (1936) developed a process of peptic digestion of antitoxic sera under carefully controlled pH conditions which accomplishes 70 to 80 per cent digestion of the plasma proteins. His

method of separating the pseudoglobulin antibody fraction is accomplished by dialysis and "salting-out" of the pseudoglobulins. For a more extensive discussion of the method employed and its effect on serum proteins the student is referred to a report by Weil, Parfentjev and Bowman (1938).

Coghill, Fell, Creighton and Brown (1940) report on a new concentration process for antitoxin which utilizes the enzyme-complex Taka-diestase. The enzyme is permitted to act on the immune horse serum for 3 to 5 days at a pH of 3.5 to 4.5 and a temperature of 37° C. Concentration is then accomplished by a modified sodium sulfate (Na_2SO_4) salting out procedure.

In all of these new methods of enzyme treatment of immune serum the antitoxin concentration is not impaired while the biologic specificity and antigenic properties have been modified. Coghill, et al., say that in addition to "despeciation" their process also produces highly concentrated antitoxins that do not elicit serious reactions upon injection into either lower animals or man.

2. SERUM SICKNESS IN THE LOWER ANIMALS.—In an excellent review of Serum Allergy, Harten and Walzer (1939) call attention to the production of serum disease in the horse by sera of cattle, man and rabbits and to serum disease in cattle produced by horse serum. Fleisher and Jones (1931, 1933, 1934, 1939) have published extensively upon serum sickness in rabbits. They describe the symptoms as elevation of body temperature, altered leucocyte picture, and redness and swelling of the ears. The incubation period was usually five or six days after a primary intravenous injection of horse serum. They noted variation in the ability of the sera of normal horses to produce "serum sickness" in rabbits.

3. MECHANISM OF SERUM SICKNESS.—While Zinsser, Enders and Fothergill (1939) seem to accept as an established fact the antibody theory of serum sickness proposed by von Pirquet and Schick (1905), Harten and Walzer (1939) state that an analysis of all available data does not support such a conclusion.

In the theory of von Pirquet and Schick it is postulated that following the injection of horse serum the antibody is formed gradually before the antigen is completely eliminated from the circulation and that the newly formed antibody reacts with the cir-

culating antigen residue, causing the symptoms of serum sickness. In support of this von Dungern demonstrated the coexistence of antigen and antibody in the blood stream of rabbits injected with horse serum. Longcope and Rackemann (1918) detected precipitins for horse serum in the blood of patients suffering from serum disease and believed they had detected a correlation between antibody production and serum sickness. Opposed to these findings according to Harten and Walzer (1939) are those of Coca (1932) that precipitins for horse serum may occur in man without the development of serum sickness and are not present (Coca, 1933), (Tuft, 1933) in the blood of patients treated with normal horse serum even though serum sickness develops.

Davidsohn (1929, 1930, 1933) carried out an extensive investigation of the titer of heterophile antibodies in the blood of individuals who had received injections of normal or immune horse serum. He seems to think there is some relationship between the heterophile antigen in horse serum and serum disease. Doubt is cast upon such a conclusion by Powell, Jamieson and Kempf (1935) who found that the removal of heterophile antigen from horse serum did not alter its capacity to produce serum sickness. Davidsohn (1938) has also called attention to an increase in titer of β isoagglutinin in patients developing serum sickness. There was no significant increase noted in α agglutinins.

Atopic reagins, according to Tuft and Ramsdell (1929), appear irregularly in the blood of individuals receiving injections of therapeutic horse serum. Doubt is cast upon their importance by the irregularity of their occurrence and the lateness of their appearance in many cases of serum sickness.

Since Zinsser, Enders and Fothergill apparently accept as fairly well established the antigen-antibody theory of von Pirquet and Schick, it would seem desirable to review and discuss the evidence which they accept as justifying such a conclusion. This evidence may be summarized as follows:

(a) The proteins in the serum injected are antigenic. Hooker (1923) described at least three antigenic fractions of horse serum.

(b) The existence of an incubation period between the injection of horse serum and the appearance of serum sickness.

(c) The appearance of antibodies for horse serum proteins in the blood of many patients receiving serum injections and an apparent correlation of their appearance with the development of symptoms.

(d) The shortening of the incubation period following subsequent injections of horse serum.

While the evidence supporting the theory of von Pirquet and Schick is very suggestive, it can only be regarded as circumstantial in nature. One should not lose sight of the fact that horse serum is foreign to man and that it, and perhaps to a lesser extent the fractions obtained from it, may possess pharmacological properties distinct from the antigenic ones. The toxic properties of antigenic substances are indicated by reactions of smooth muscle to toxic doses of horse serum and other antigens described by Dale (1913) and Sherwood and Stoland (1923) as distinct from antigenic properties. The Brodie reaction (1900) in cats is usually ascribed to the presence of acetyl choline in normal serum. Incidentally, it has been shown in this laboratory that the Brodie reaction resembles the anaphylactic response in cats as determined by blood pressure changes (Kabler and Sherwood, 1938). These examples are given to emphasize the point that serum may have toxic as well as antigenic properties. Another example which may bear more directly upon serum disease is the pulmonary permeability and vagus irritability changes produced in dogs by the injection of horse serum. Manwaring, Chileote and Hosepian (1923) who first described the change in pulmonary permeability that follows the injection of horse serum attributed it to anaphylactic sensitization. That the phenomenon is not an antigen-antibody reaction is indicated by the work of Sherwood and Stoland (1930) and Stoland, Sherwood and Woodbury (1931).

The evidence they presented may be summarized as follows:

(a) The phenomenon is observed in animals that show no other evidence of sensitization to horse serum.

(b) It can be demonstrated by perfusion with Locke's solution from which antigen is omitted.

(c) It can be demonstrated in desensitized dogs.

(d) The incubation period is shorter than in anaphylaxis.

(e) The chronaxie studies indicate that following the injection of horse serum there develops an increase in irritability of the

vagus nerve which correlates with increased permeability of the tissues but not with the classical anaphylactic sensitization.

That the existence of an incubation period is not in itself proof of the antigen-antibody mechanism of serum sickness is suggested by the fact that periods varying from hours to days elapse between the injection of various drugs, tuberculin and toxic substances and the appearance of symptoms. In drug allergy there is frequently an incubation period of 6 or more days and symptoms that in part at least may resemble serum sickness. As in serum sickness the incubation period is shortened by subsequent injections of the exciting agent. In the tuberculin type of skin reactions and in positive Schick tests there is an incubation period, so to speak, of 24 to 48 hours or longer. All efforts to show that drug allergy and allergy due to infection are mediated by an antigen-antibody mechanism have failed. In the case of the positive Schick test it is the absence of antibodies that permits the toxin to act upon the tissues. To explain the short incubation period occasionally observed in serum sickness Zinsser, Enders and Fothergill suggest the possibility of intrauterine or intestinal sensitization but offer no statistical data to support such a correlation.

Since the incubation period for serum sickness is frequently seven to ten days and since horse serum contains antigenic proteins one might expect that frequently the correlation would be noted between the appearance of antibodies and the development of symptoms. This could easily be a coincidence. It is not likely that the union of antigen and antibody in the blood stream would cause symptoms as this phenomenon is offered as an explanation for the failure of symptoms to occur in sensitized animals. It has been called "masked anaphylaxis." Dean (1931) has suggested that perhaps the reason that antigen and antibody, at times, coexist in the blood without the formation of a precipitate is the absence of optimal ratios of concentrations of each. Furthermore the symptoms in serum disease are not those of anaphylaxis. Contrast the fever of serum disease with the subnormal temperature of anaphylaxis, the absence of pulmonary symptoms in serum disease and their presence in human anaphylaxis. In the rabbit, note that in serum disease there is an elevation of temperature, swelling and hyperemia of the ear but no cardiac symptoms while

in rabbit anaphylaxis there is lowering of the temperature, complete blanching of the ear and when death occurs it is due to right heart failure.

The shortening of the incubation period in man following subsequent injections is not proof of anaphylactic or antigen-antibody nature of serum sickness. As mentioned above a similar phenomenon occurs in drug allergy. The accelerated reaction is not an immediate one as in anaphylaxis but possesses a delay of six hours or more. The incubation period may be short because of the toxic effect of the first injection upon the tissues permitting a more rapid entry of substances into the cells.

While we are inclined to regard serum sickness as a manifestation of some form of allergy, we agree with Coca that an antigen-antibody mechanism has not as yet been established.

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CHAPTER XVII

BIOLOGICAL AND ANTIGENIC SPECIFICITY

Immunological Specificity.—In the previous pages attention has been directed to the phenomenon of immunological specificity wherein a specific antigen not only stimulates the physiological production of specific antibodies but reacts only with the antibodies thus formed. Landsteiner (1936, p. 5) points out that specificity is not an entirely absolute quality but that there is always some overlapping. Loeb (1916), Wells (1929), Zinsser, Enders and Fothergill (1939) and others have called attention to the general biological significance of specificity and have reviewed the literature and offered experimental evidence pointing to a chemical basis for all specificity.

Biological Specificity.—Experience in animal breeding and in cross fertilization has shown that success is attained only when eggs and spermatozoa of the same or very closely related species are used.

SPECIFICITY AND INCOMPATIBILITY OF SPECIES.—In discussing the incompatibility of species not closely related, Loeb mentions the rigid specificity requirements for successful skin grafting or organ transplantation. It is the experience of every plastic surgeon that the best results are obtained when the skin from the patient's own body is used or from members of the family who belong to the same blood group. In any event skin from a different species cannot be used for a successful graft. The same applies to organ transplantation.

SPECIFICITY AND FERTILIZATION.—Loeb also calls attention to the specificity requirements in the fertilization of an egg by a sperm. In this process many phenomena analogous to those observed in immunity are encountered. The first of these is positive chemotaxis. Pfeiffer showed that fern spermatozoa are deviated in their course when they approach an archegonium containing an egg, and that the attraction is sufficient to cause the sperm to enter the archegonium and permit contact and ultimate fertilization of the egg. Loeb says that it is a very common experience

“that spermatozoa become very active when they reach the neighborhood of an egg.” Lilly has shown this to be a specific effect or activation. When certain spermatozoa such as those of the California sea urchin, *Strongylocentrotus purpuratus*, or of certain annelids are put into sea water which has been in contact with the corresponding eggs, specific clumping or agglutination of the spermatozoa occurs.

UNION OF EGG AND SPERM.—The fact that in fertilization, the sperm enters the egg and only the eggs of the same or closely related species, indicates not only specific invasive power on the part of the sperm but also specific susceptibility on the part of the egg for the same sperm. Toward the spermatozoa of other species it exhibits a general resistance. After fertilization with suitable spermatozoa has taken place the egg becomes immune to invasion even by spermatozoa of the same species.

Loeb calls attention to the similarity of fertilization or union of sperm and egg to the phenomenon of phagocytosis. The spermatozoan enters through a protoplasmic process (fertilization cone) which is comparable to the pseudopods of ameboid cells. As a result of the entrance of the spermatozoan, in the case of the sea urchins studied by him, the egg develops a membrane which acts as a barrier to the entrance of any more spermatozoa. This is obviously a specific defensive mechanism. Loeb has been able to modify these various mechanisms, and to a large extent control them by altering the salt content and pH of the sea water. He concludes that the biological specificity in fertilization is chemical in nature.

SPECIFICITY AND GENETICS.—The exactness with which geneticists can predict the appearance of certain characters in the offspring of inbred strains of the grasshopper or white rat, plants, etc., indicates that specific reactions are at work and that the phenomenon of specificity is of broad biological significance.

PRODUCTION OF SPECIFIC HORMONES.—In the animal body specific chemical substances such as thyroxin, insulin, cortin, adrenalin, etc., are normal physiological products and each brings about certain specific physiological reactions. The constancy of production and the specificity of bacterial enzymes has long been

known and used in bacterial identification. That this specificity is definitely due to chemical structure is now generally accepted.

Thus it will be seen that immunological specificity is but another example of a phenomenon quite common in nature.

Immunological Specificity.—Gruber and Durham (1896) were really pioneers in the study of immunological specificity. They discovered, while working with bacterial antigens, specific and group agglutination. They noted that when they obtained an immune serum that agglutinated in high titer suspensions of the organisms used in the antigen, this same immune serum frequently agglutinated closely related bacteria at a lower titer. For example, an antityphoid immune serum that would agglutinate only suspensions of *E. typhosa* when the serum was diluted 1:10,000 might agglutinate suspensions of closely related organisms when diluted to only 1:100 or 1:200.

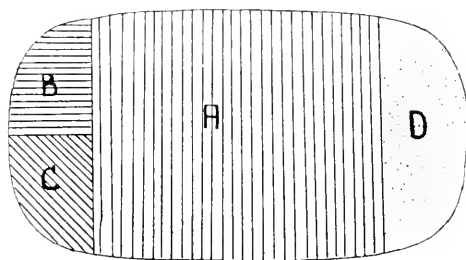


Fig. 14.—Durham's conception of the multiplicity of cellular antigens. A, Major antigen. B, C, and D, Minor antigens.

DURHAM'S EXPLANATION—MULTIPLE ANTIGENS.—Sometimes group agglutination at a higher dilution could be demonstrated. By using high titered serum they could demonstrate species specificity, but the group reactions did not always indicate closely related species. Durham attempted to explain the group reactions as due to the presence of identical antigens of different amounts in different bacteria. In his opinion, each species of bacteria contained a chief antigen that was species specific and a number of minor antigens that might be present in closely related organisms. This concept is indicated in Fig. 14. Here A represents the chief antigenic constituent of the cell while B, C, D, and E represent minor antigens. Any one of these might be found in some other closely related organism in different amounts. Varying amounts

of antibodies would be produced for each kind of cell antigen; the amount of antibody response would correspond to the amounts of various antigens present. The major antigen would lead to the production of a major or chief agglutinin, while minor agglutinins would be produced for the corresponding minor antigens. Landsteiner (1936) in an excellent discussion of antigens considers cellular antigens as forming a mosaic pattern. This has been discussed in the preceding chapter.

PRECIPITINS AND SPECIFICITY.—Shortly after Gruber and Durham's discovery of specific and group agglutination of bacteria, Kraus (1897) reported that filtered extracts of bacteria would give specific precipitates when mixed with their homologous immune serum. Two years later Tchistovitch (1899) found that blood serum (eel) when used as an antigen led to the production of precipitins in rabbits. There followed, according to Pick (1904), extensive investigations of antigens as to their nature, method of preparation, chemical and physical structure, and specificity. Nuttall (1904) used quantitative precipitin tests in an extensive study of the zoological relationships in the animal kingdom. His results were in harmony with the accepted zoological relationships which had been based upon morphology. This indicated that the process of evolution included biochemical as well as anatomical and morphological changes.

PRECIPITINS AND SPECIES RELATIONSHIP.—While the work of Nuttall (1904) and others indicated that the precipitin test could be used to identify the blood proteins of a species (species specificity parallels immunological specificity), a discovery had been made by Landsteiner (1902) which seemed to be at variance with this. He found that he could differentiate 3 or 4 groups within the human species by means of isohemagglutination.

SEROLOGICAL TYPES WITHIN A SPECIES.—Thus while Nuttall and others had shown that the serum proteins of all members of a species were alike antigenically, Landsteiner had shown that the red cells of different groups of individuals within the species (human) were antigenically unlike and that the dissimilarity probably had an hereditary basis.

BACTERIAL TYPES WITHIN A SPECIES.—In 1909, Neufeld described antigenic differences in a bacterial species, the pneumo-

coccus, and since then Dochez, Avery, Blake and others have found, by means of agglutination, antigenic types for the meningococcus, streptococcus and many other kinds of bacteria. Thus it will be seen that at least two kinds of specificity can be demonstrated by immune reactions, one that applies to species and the second to type variation within a species.

SERUM PROTEINS VS. CELLULAR ANTIGENS.—Landsteiner (1928) has called attention to the fact that species relationships are indicated by a study of the serum proteins, while type differentiation within a species is determined by studies of the cellular antigens peculiar to the species. Wells (1929) considers that even the serum proteins show two kinds of specificity and this is borne out by the work of Nuttall and others.

Ehrlich (1910) says that his side chain theory of immunity implied that antigenic specificity was dependent upon the chemical constitution of the antigen. Subsequent research has apparently confirmed this concept.

Formerly it was thought that only complete proteins were antigenic but it is now agreed that a few bacterial polysaccharides (e.g., Type I pneumococcus) and perhaps a few lipid-carbohydrate complexes may stimulate specific antibody formation. Wells (1929) summarized the properties of antigenic protein substances somewhat as follows:

Properties of an Antigen.—They are all complete proteins in a colloidal state, soluble in the fluids of the animal body and possessing a necessary number and kind of aromatic amino-acids. To elicit antibody production they must be introduced beneath the "epithelial coverings of the body," i.e., into the tissues of an animal not possessing similar antigens in its body proteins. (It is now generally believed that many of these properties are possessed by all antigens whether protein or carbohydrate.)

To appreciate the basis for the above conclusion of Wells and others, it is necessary to compare the results of chemical and immunological investigations of both animal and plant proteins as well as experimentally modified proteins. Similar comparisons should also be made for the carbohydrates and lipoids.

Animal Proteins.—*Proteins of the animal body* consist of the proteins of the blood, tissues, and normal secretions of the body.

Largely through the work of Leblanc (1901), Hektoen (1918), Dale and Hartley (1916), Doerr and Berger (1922), Lewis and Wells (1927) and others it has been shown that blood plasma contains at least five chemically different proteins that are also antigenically different. These plasma proteins are euglobulin, pseudoglobulin, albumin, seromucoid and fibrinogen. Of all these proteins only one, fibrinogen, lacks species specificity. However, even with it there is a quantitative difference that is definite since the maximum precipitate occurs between an antifibrinogen immune serum and the species fibrinogen used to produce it. Goodner (1925) found that beef serum was antigenically different from beef chondromucoid or beef submaxillary mucin.

RED CELL PROTEINS.—Hemoglobin present in the red cells is both chemically and antigenically different from the proteins of the plasma. Even the globulin from the red cells shows antigenic differences from the serum globulin. The red cells also contain lipoids and carbohydrates which form compounds with some of the red cell proteins, giving rise to chemical and immunological differences. This has been discussed in the chapter on Blood Grouping.

TISSUE PROTEINS.—Downs (1925) has shown that tissue fibrinogen is species specific antigenically, which is interesting in view of the fact that Hektoen and Welker (1925, 1927) showed that blood fibrinogen does not show species specificity.

Goodner (1925) found marked antigenic differences between the mucins obtained from beef submaxillary glands and from the hog stomach. He calls attention to the fact that the conjugated proteins are relatively poor antigens and that their antigenic efficiency bears some relationship to the amount of protein present in the conjugate. This is in harmony with other experimental results which show that with a few exceptions tissue proteins do show species specificity.

ORGAN SPECIFICITY.—Pfeiffer (1910) even claimed that antigenic difference could be detected between the proteins of the liver, spleen, kidney, etc., but Pearse, Karsner, and Eisenbrey (1911) failed to confirm Pfeiffer's claims. Later Fleischer (1920, 1921) seems to have demonstrated some slight difference.

LENS AND TESTICULAR PROTEINS SHOW NONSPECIES SPECIFICITY.—Of the tissue proteins lacking species specificity, those of the

crystalline lens of the eye and of the testis are possessed by all species, while Forssman's antigen and somewhat similar substances recently described by Landsteiner and Levine (1932) are peculiar to only a limited number of species of animals as well as a few species of bacteria. Since Forssman's antigen is a lipid-carbohydrate-protein complex, or at any rate a hapten-protein combination, it is possible that the hapten portion is the fraction common to different species, etc. In regard to lens protein, Kraus, Doerr and Sohma (1908), Uhlenhuth (1910), Hektoen (1921, 1927) have found that protein from the crystalline lens of the eye is antigenically and chemically the same in all species. It even functions as an antigen when injected into the same species. Von Dungern and Hirschfeld (1910) claim to have demonstrated similar nonspecies specificity for testicular protein, although they did not find such marked organ specificity as exists in the case of lens protein.

KERATIN, MUCIN, ETC., LACK SPECIES SPECIFICITY.—Wells (1929) says that "it is altogether reasonable that lens proteins, keratin, mucin and other proteins whose function is not metabolism, should be nonspecific. Each of these proteins has quite the same function to perform in every species, and is set off from the active tissues to perform it."

THYROGLOBULIN.—Hektoen and Schulhof (1923) found that thyroglobulin is antigenically different from the other proteins of the same animal from which it is obtained but antigenically similar to thyroglobulin of other species.

MILK PROTEINS.—*Proteins of normal body secretions.* Milk is a normal secretion of the mammary gland and contains proteins, carbohydrates, fats, and other ingredients. Casein, the principal protein, is chemically identical and antigenically similar for all species, but in each species its chemical and antigenic properties are different from other body proteins. On the other hand, the globulin present in milk is chemically and antigenically similar to the serum globulins of the species and shows the same species specificity as the serum globulins.

ANTIGENIC COMPONENTS OF THE EGG.—Wells (1911) and Hektoen and Cole (1928) have demonstrated five antigenic components

of hen's eggs corresponding to five chemically different proteins that have been isolated and studied. Wells has also called attention to the chemical and antigenic similarity between the crystallized albumen of the egg from both the hen and the duck. Goodner (1925) found some antigenic relationship between two glucoproteins of the hen's egg.

BIOLOGICAL AND IMMUNOLOGICAL RELATIONSHIPS AMONG AMEBÆ.—Heathman (1932) made extensive immunological studies of a number of strains of free-living and pathogenic amebæ. Her results corroborated the morphological classification of Schaeffer (1926). Her results indicated that there are both a highly specific and a common radical present in the antigen which are responsible for the specific and nonspecific reactions. Sherwood and Heathman (1932) found two haptens present in *Entamoeba histolytica*. The chief one was alcohol soluble and a second was soluble in saline, as were the haptens present in the free-living amebæ.

VEGETABLE PROTEINS.—Wells and Osborne (1916) and others working in Wells' laboratory have made extensive studies of the vegetable proteins and found them especially valuable for comparative chemical and antigenic investigation. They isolated gliadin from both wheat and rye and found them chemically and immunologically identical. They could differentiate by immunological methods proteins such as hordin of barley and glutenin of wheat not only from each other but also from gliadin. They also could detect by both chemical and immunological methods a protein legumin, common to the seeds of peas, vetch, lentil and horse bean. In their studies of the proteins of leguminous seeds, they isolated from each species two globulins, α and β , which are chemically and immunologically different. This was of especial interest since Wells had also shown that globulins from different beans may show chemical and immunological similarity.

ALGÆ.—Steinecke (1925) carried out extensive immunological and biological comparisons of algae, etc., with an idea of throwing light upon evolutionary development. He concluded that it is with the descendants of the euglenas that one is to look for the foundation of the animal kingdom. Elmore (1928) found that

Euglena gracilis Klebs is not a homogeneous group but consists of two distinct antigenic types. These correlate with growth characteristics.

ANTIGENS IN FUNGI.—Hektoen (1901) noted antigenic properties for a strain of *Blastomyces dermatitis*. Recently Dulaney (1930) confirmed this work, but could not demonstrate species specificity in the blastomyces. She says that "slightly positive results were obtained with closely related yeasts and specific results were obtained upon titration of the serums."

In this connection it is interesting to note that Mueller and Tomesik (1924) obtained a soluble specific substance from yeast and found it to be identical with the "yeast gum" of Salkowski (1894). It yielded specific precipitates with homologous immune serum but was found to be nonantigenic. This work has been extended recently by Tomesik (1930). Kesten, Cook, Mott, and Jobling (1930, 1931) have isolated specific polysaccharides from strains of monilia.

BACTERIAL ANTIGENS.—While this subject will be discussed in Chapter XIX, attention can be called here to the fact that they, like the algae, fungi, amebae and red blood corpuscles, represent cellular or protoplasmic antigens exceedingly complex in nature and in which haptens play an important rôle in the serological reactions. By agglutination and absorption tests it has been possible to demonstrate species specificity for some species, e.g., *E. typhosa* while for others like *Esch. coli* only strain specificity has been demonstrated as yet.

Only a small amount of albumin or globulin has been found in the bacterial extracts, the bacterial cell substance containing for the most part nucleoproteins. A continuation of the study of bacterial proteins such as the P_1 and P_2 fractions obtained by Furth and Landsteiner and of the cell haptens gives promise of a better understanding of bacterial antigenic specificity.

Structure and Properties of Proteins.—When one looks at the proteins from the standpoint of their chemical structure and biochemical properties, one discovers a number of reasons why most antigens are probably protein in nature and how their struc-

ture may account for specificity. In the first place, they are colloids, chemically different yet possessing physical properties and chemical constituents similar to those of the tissues of the animal which give rise to antibodies. Second, the enormous number of possible combinations of the 20 or more amino acids might well account for the equally large number of species of living organisms; third, they possess the property of linkage with many different kinds of chemical elements that could account for further specific diversity both in their character and also in their reactivity; and fourth, the different proteins are quite constant in their chemical composition.

IMPORTANCE OF PHYSICAL AND CHEMICAL CHANGES IN ANTIGEN.—Zinsser calls attention to the fact that true antigens are composed of large molecular aggregates incapable of diffusion through a membrane and that this is probably a necessary state to induce antibody response. When irreversible coagulation of an antigenic protein occurs, it becomes antigenically inert. Wells (1929) cites the irreversible coagulation of egg albumen by alcohol as an example of this.

The same antigen coagulated by heat tends to redissolve and retains its antigenic property only so far as it goes back into solution.

Wells (1909) further showed that when an antigen such as egg albumen is treated with hydrochloric acid, acid albuminate is formed without impairment of the antigenic properties. On the other hand, if the egg albumen is treated with alkalis with the formation of alkali albuminate, all antigenic properties are lost. This, according to Dakin (1912, 1913), is associated with a loss of optical activity and also the capacity to be acted upon by enzymes and is called "racemization."

It should be noted that racemized antigens, however, retain their solubility and coagulability and all of their amino acid constituents. The change, according to Wells (1929) and Dakin, is in those amino acids that have their amino groups linked to a carboxyl group within the protein molecule. "The terminal amino acid groups containing a free carboxyl group remain unchanged." (Wells and Dakin.) They speak of this change as

enolization and say that it consists of the loss of asymmetry by the α carbon atom. Wells* illustrates this as follows:



It will be observed that in the ketone form the α carbon is linked to four different groups; this makes of it an asymmetric carbon atom. In the enol form it is linked to three, and hence is no longer asymmetric and can now form equal amounts of two isomers which accounts for its loss of optical activity, etc.

ENZYME ACTION DESTROYS ANTIGENIC PROPERTY.—It has been quite well established that when proteins are acted upon by enzymes their antigenic properties are diminished and entirely lost during the process of digestion.

RELATIONSHIP OF DIGESTIBILITY AND ANTIGENIC PROPERTY.—Since racemized proteins are neither antigenic nor digestible by enzymes and since complete proteins possess nondiffusible molecular aggregates and are both antigenic and digestible, it has been suggested that for antibody production the antigen must reach the surface of the tissue cells and call forth extracellular enzymes. It should be remembered that the molecular aggregates of an antigen may be phagocytized, so to speak, by the reticulo-endothelium and digestion occur within food vacuoles. Antibodies could be returned to the general circulation by a process the reverse of that of phagocytosis.

Landsteiner does not regard the lack of digestibility of racemized proteins as the reason for their lack of an antigenic property but thinks it is due to their lack of a chemical structure necessary to stimulate antibody formation.

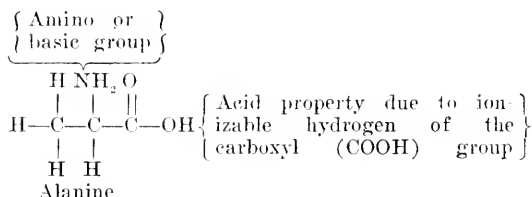
POSSIBLE NUMBER OF COMPOUNDS FORMED BY AMINO ACIDS.—Abderhalden has calculated, according to Wells (1929), "that 20 amino acids could form 2,432,902,008,176,640,000 different compounds, and this without including the enormously greater number that might be made by varying the proportion of the different

*Wells, H. G.: The Chemical Aspects of Immunity, Reinhold Publishing Corporation.

amino acids in a single protein." Wells regards this as strong evidence that immunological and biological specificity depend upon the proteins. Subsequent work has shown, however, that *a few* apparently nonprotein polysaccharides or carbohydrate-lipoid complexes can stimulate specific antibody production.

IMPORTANCE OF AROMATIC AMINO ACIDS IN PROTEIN ANTIGENS.—Starin (1918) working in Wells's laboratory found that gelatin, which is a derived protein lacking tyrosine, is nonantigenic. From an extensive chemical examination of protein antigens Obermayer and Piek (1906) conclude that aromatic amino acids are essential to all antigens and conversely that compounds made up of aliphatic amino and diamino acids are not antigenic.

ACID AND BASIC GROUPS OF AMINO ACIDS.—It will be remembered that the aliphatic amino acids have an open chainlike structure while the aromatic amino acids have somewhere in their structure a closed chain. The benzene ring is quite common. Each possesses one or more amino (NH_2) groups and also a carboxyl (COOH) group. The former endows it with basic properties while the latter, through its ionizable hydrogen, gives it acid properties. Normally these tend to balance each other. If the basic properties are removed, through linkage of some ethyl or methyl group to the nitrogen of the amino group by replacement of a hydrogen, the acid property is increased through this diminution of the basic property. In a similar way the acid property may be reduced and the basic property increased. These basic and acid groups are shown in the following formula for alanine:



ALIPHATIC AND AROMATIC AMINO ACIDS.—The student should remember that the aliphatic or open chain amino acids can be acetylated or methylated but cannot be diazotized. This will be referred to again in the next chapter. The aromatic amino acids, on the other hand, can be methylated, acetylated, azotized or

diazotized and nitroso compounds can also be prepared from them. This is largely because the diazo (—N=N—) compounds link readily to the nucleus, provided it possesses an OH or an NH_2 group to favor linkage. This will also be made more clear in the next chapter. A few of the aromatic amino acids are tyrosine, phenyl-alanine, tryptophane and histidine. Their structural formulae and importance in antigens will be apparent from a study of the chapter on modified antigens.

Zozaya's Nonprotein Antigen.—Zozaya (1932) reports that a colloidal solution of collodion particles which had been made to absorb protein-free carbohydrate haptens of the pneumococcus functioned as an antigen. This has been referred to in a previous chapter.

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CHAPTER XVIII

MODIFIED AND CONJUGATED ANTIGENS*

Early Work on Modified Antigens.—The first work on modified antigens was that of Obermayer and Pick (1906). They made the important observation that chemical *alteration* of proteins leads to the appearance of new serological properties. They introduced iodine and NO_2 into proteins and thus formed iodoproteins and xanthoproteins, respectively. Antibodies produced by injecting these altered proteins gave visible (precipitin) reactions with any protein similarly altered but not with the original unaltered protein. It was the general belief at that time that the capacity of inciting antibody production as well as of reacting in vitro with antibodies was the property solely of proteins. This notion was retained long after the publication of Obermayer's and Pick's papers and the idea that antibodies could react with simple chemical substances was not considered at all. It is now known that antibodies for xanthoprotein or iodoprotein will *not react* with NO_2 or I_2 (or KI), respectively, but are specific for chemically modified portions of the proteins. This should be kept in mind because it really differentiates the discoveries of Landsteiner from those of Obermayer and Pick.

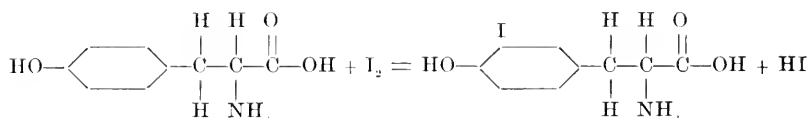
Early Work on Conjugated Antigens.—Landsteiner began by confirming Pick's work and extending it to include acetylated and methylated proteins. He then started a new line of investigation, namely, an attempt to *manufacture* antigens by combining simple substances with proteins. From this work, he says, two unforeseen facts emerged.

First, that it is possible to produce antibodies which are specific for substances of known constitution. These results were published more than ten years after the studies of Obermayer and Pick. The second unforeseen result (1920) was that by means of a new method, namely, the "inhibition reaction" antibodies were

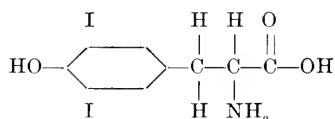
*Dr. R. Q. Brewster of the Organic Chemistry Department collaborated with the author in the preparation of part of this chapter.

shown to be capable of reacting with very simple crystalline substances such as benzoic acid, etc. (Landsteiner, *Specificity of Serological Reactions*, 1936, p. 118). Landsteiner has also discovered the importance of spatial relationships and of the relative position of chemical groups in determining specificity. For a better understanding of the early work of Obermayer and Pick and the distinction between their altered antigens and the new conjugated antigens of Landsteiner and others, structural formulae are employed in the following discussion:

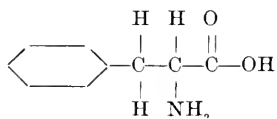
Altered or Modified Antigens.—**IODIZED PROTEIN.**—When iodine is added to a protein it very likely replaces the hydrogen adjacent to the hydroxyl ($-\text{OH}$) group of some aromatic nucleus; thus if tyrosine is in the protein, the reaction could be illustrated as follows:



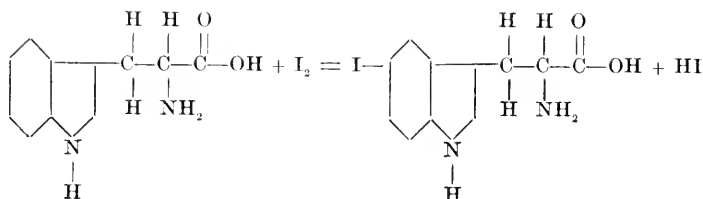
If an additional mole of iodine is added, the other hydrogen adjacent to the hydroxyl ($-\text{OH}$) group may be replaced to give a compound of the following formula:



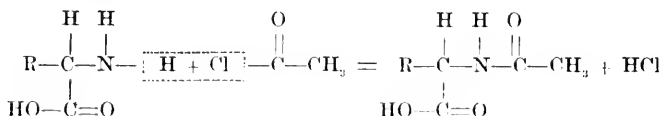
In the case of phenylalanine it is hard to introduce iodine since there is no hydroxyl or other activating group on the ring, as will be seen from the following formula of phenylalanine:



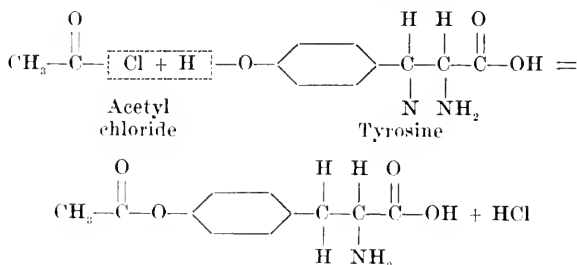
IODOTRYPTOPHANE.—Tryptophane has not been extensively studied, but it is regarded as iodizable, probably through the replacement of a hydrogen on the nucleus as follows:



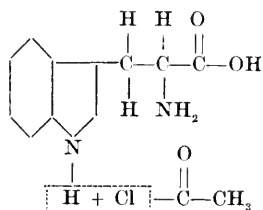
ACETYLATED PROTEINS.—The acetylated proteins have been extensively studied by Landsteiner. In such compounds the acetyl group doubtless combines with the hydroxyl ($-\text{OH}$) or amino ($-\text{NH}_2$) groups of the protein molecule. Thus any amino acid might be acetylated in the following manner:



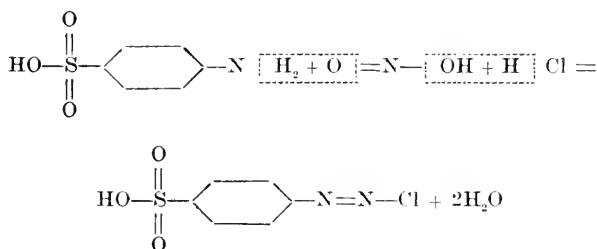
In the case of tyrosine the acetyl group might be attached to either the hydroxyl ($-\text{OH}$) or amino ($-\text{NH}_2$) group, or perhaps to both. The hydroxyl linkage is illustrated as follows:



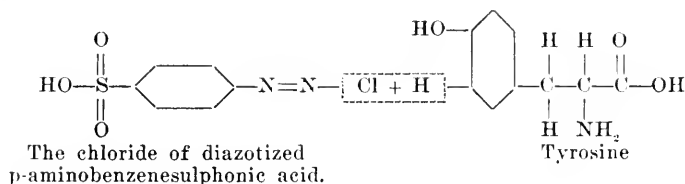
In the case of tryptophane the reaction would be the replacement of either the single hydrogen on the nitrogen of the indole ring, or of the hydrogen in the amino ($-\text{NH}_2$) group on the side chain.



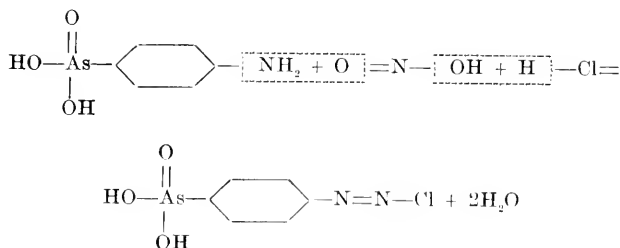
Conjugated Antigens of Landsteiner.—DIAZO COMPOUNDS.—By diazotization is meant the treatment of an aromatic amino compound with nitrous acid and hydrochloric acid in the cold. As a rule Landsteiner diazotized compounds that he wished to add to his proteins. It is very difficult to determine just where the diazonium chloride would couple to the protein molecule. From the standpoint of pure organic chemistry, however, the coupling of the diazonium chloride with the tyrosine would be most logical, since this is in direct accord with the common procedure for making azo dyes. Students of organic chemistry will recall that phenols couple with phenyl diazonium chloride with the greatest of ease. For example, Landsteiner diazotized p-aminobenzenesulphonic acid by adding nitrous acid (NHO_2) and hydrochloric acid (HCl) in the cold; water split off and the diazonium chloride resulted:



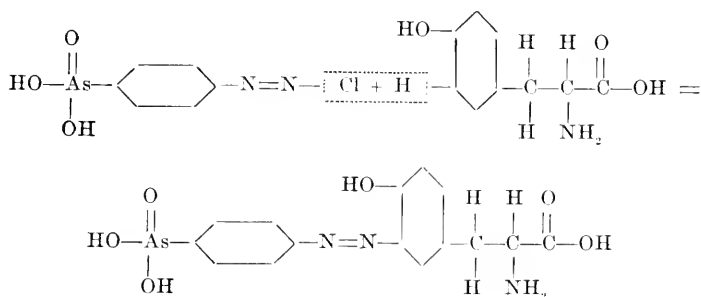
COUPLING WITH A PROTEIN.—This diazonium chloride could be coupled to a protein since it will unite with tyrosine and perhaps also other parts of the protein by replacing the hydrogen next to the hydroxyl ($-\text{OH}$) group on the benzene ring, as is illustrated below with tyrosine:



ARSONIC HAPTENS.—In the same way he diazotized p-aminobenzenearsonic acid and coupled it with proteins forming a new antigenic compound:

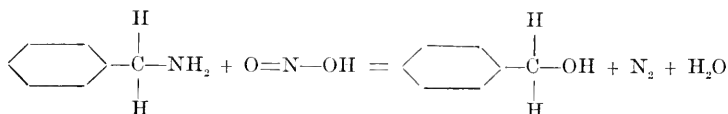


This p-aminobenzenearsonic diazonium chloride could react with aromatic amino acids of the proteins replacing a hydrogen on the nucleus, as was the case with the sulphonic acid derivatives. Using tyrosine again as an illustration we have:



ARSONIC PROTEIN COMPOUNDS.—The compound of protein and the p-aminobenzenearsonic acid is a new antigen for which specific antibodies are produced.

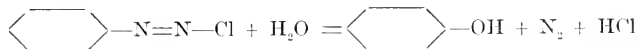
ALIPHATIC SIDE CHAINS NOT DIAZOTIZABLE.—When an amino ($-\text{NH}_2$) group is in the side chain of a compound, i.e., not attached to a carbon atom in a benzene nucleus or ring, as in benzyl amine $\langle \text{C}_6\text{H}_5 \rangle - \text{CH}_2\text{NH}_2$, that amino group is in general not diazotizable, the reaction with nitrous and hydrochloric acids under such conditions giving the corresponding alcohol, free nitrogen (N_2) and water. This may be shown as below, using benzyl amine:



It is important that the diazotization of aromatic amino groups, i.e., amino groups attached directly to a carbon atom in a benzene nucleus or ring as in aniline $\text{C}_6\text{H}_5\text{NH}_2$, be carried out in the cold, for otherwise hydrolysis ensues with the formation of hydrogen chloride (HCl), free nitrogen (N_2) and the corresponding phenol as shown below :

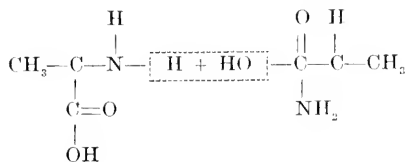


In a cold solution, this is the only reaction, but if the solution is warm a second reaction takes place which is the hydrolysis reaction yielding the phenol :



thus giving in the end products analogous to the reaction in the aliphatic series.

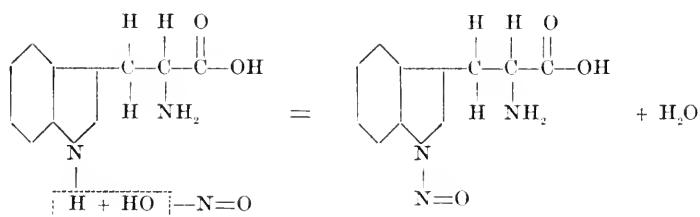
PROTEIN STRUCTURE.—It might be thought that the amino groups on the side chain of the amino acids constituting the protein would be attacked by the diazotization thus giving hydroxy acids rather than amino acids, but it will be remembered that the protein molecule is conceivably built up by condensation of an amino group of one amino acid molecule with the carboxyl ($-\text{COOH}$) group of another amino acid molecule as shown :



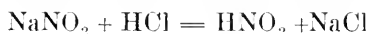
Alanyl-alanine, a dipeptid.

until finally a compound of high molecular weight and having emulsoid colloid properties is formed. In this type of combination all but a few of the side chain amino groups of the amino acids constituting the protein are protected against the action of the nitrous acid.

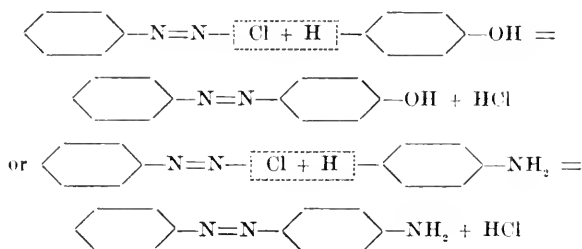
NITROSO COMPOUNDS.—For diazotization it is also necessary that the group be an amino ($-\text{NH}_2$) group and not an imino ($=\text{NH}$) group. In the case of tryptophane the $=\text{NH}$ group in the ring is not diazotizable, $-\text{NH}_2$ being required for diazotization. However, nitroso compounds can be formed here by reaction with nitrous acid in the following manner:



Azo Dyes.—For diazotization it is customary to use sodium nitrite and hydrochloric acid, the reaction between sodium nitrite and hydrochloric acid that yields nitrous acid being as follows:



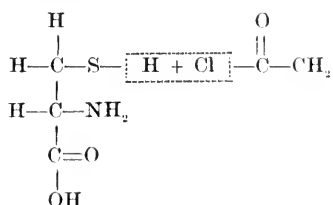
The preparation of azo dyes is accomplished by coupling diazonium chlorides in solution with phenols or aromatic amino compounds,



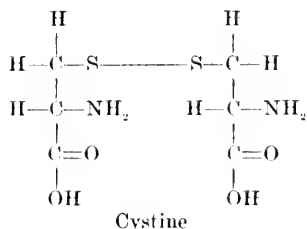
In the protein a similar coupling can be accomplished whenever the aromatic nucleus has an $-\text{OH}$ or an $-\text{NH}_2$ in it, as has been illustrated heretofore.

CYSTEINE AND CYSTINE.—In the case of cysteine, diazotization would not be possible, nor would it be in the case of cystine, since neither of these two compounds contains the aromatic nucleus.

However, acetylation or methylation of cysteine might be possible through replacement of the hydrogen on the sulphur as follows:



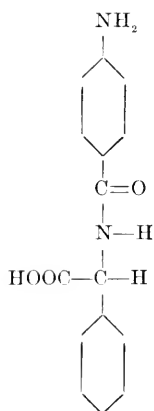
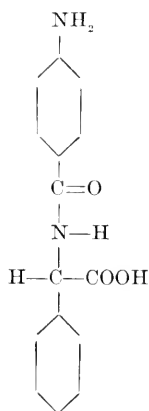
Since there is no replaceable hydrogen on either of the sulphurs of cystine, it is not possible to acetylate or methylate as shown by the structural formula:



The chance for acetylation or methylation of the amino group of either of these two compounds or of any amino acid when combined in a protein is extremely remote, as explained under protein structure.

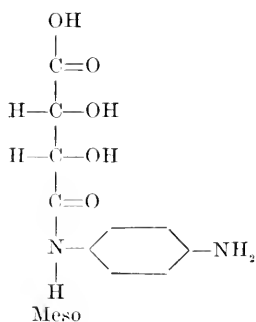
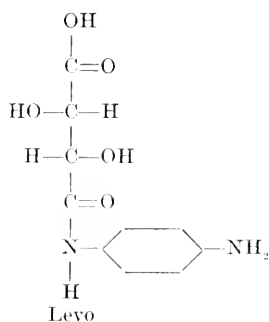
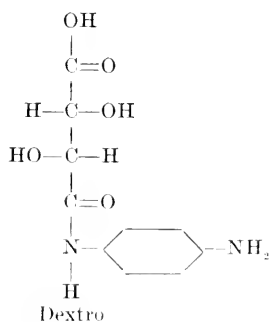
STEREISOMERS OR SPATIAL RELATIONSHIPS.—Landsteiner calls attention to another interesting factor, spatial relationship, that may influence specificity, especially in the natural antigens containing carbohydrates. This type of specificity is due to the configuration around an asymmetric carbon atom. He finds when he couples stereoisomers to a protein such as horse serum, by diazotization, that each optical isomer-azo-protein complex constitutes a distinctly specific antigen for which corresponding antibodies can be produced. This is another example of hapten specificity.

When phenyl glycine is treated with p-aminobenzoyl chloride there results phenyl-p-aminobenzoylamino acetic acid, of which Landsteiner prepared the two optical isomers as shown:

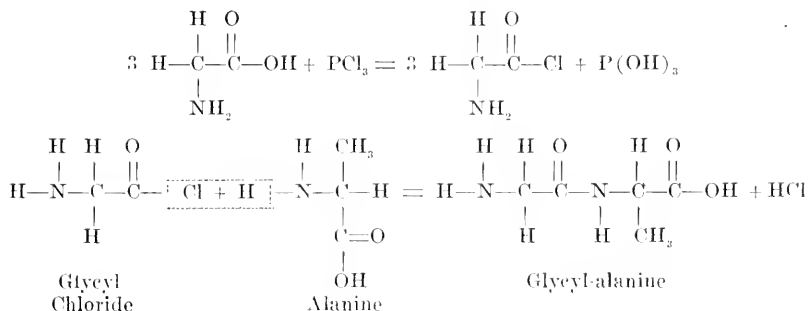


Here the mere difference in relative positions of a hydrogen atom and a carboxyl group suffices to alter the serological reactivity.

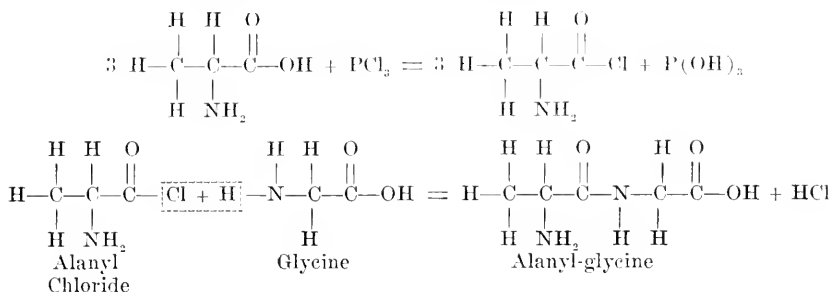
Landsteiner also succeeded in obtaining immune sera which differentiated sharply the three antigens obtained by coupling dextro-, levo- and meso-tartaric acids to proteins such as horse serum through diazotization of the corresponding p-aminotartrilic acids, formulae for which are shown below:



POSITION ISOMERS.—Landsteiner also says that the relative positions of groups are quite important in determining specificity. An example may be seen in a consideration of glycyl-alanine and alanyl-glycine. It would be difficult to differentiate them chemically but they should be biologically unlike since their position relationships are different. The glycyl-alanine is formed as follows:

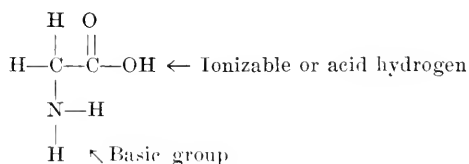


and in this compound the terminal carboxyl ($-\text{COOH}$) group is the one from the alanine molecule. Now if this process is reversed, one may produce the alanyl-glycine with the terminal acid group that of the glycine molecule, thus,

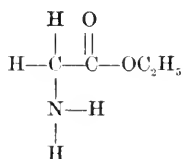


Linkage With Salt-Forming Groups.—While Pick and others regard the linking of the various haptens to the amino acids as replacement of hydrogen either on the ring or on an amino group attached to the ring, Landsteiner considers that linkage with salt-forming groups takes place in the formation of many of these new antigens. In some instances this might be explained as follows:

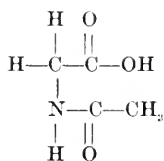
Removal of Acid Properties of an Amino Acid.—If one considers a simple amino acid like amino acetic acid, the formula for which is



it will be observed to have both a basic ($-\text{NH}_2$) and an acidic ($-\text{COOH}$) group. Thus, reacting as an alkyl amine it yields salts with acids and as an acid forms salts with bases. If the ionizable hydrogen is replaced by an ethyl group to form the ethyl ester, then the acid properties are destroyed and the basic properties are dominant, so that it might unite through the basic ($-\text{NH}_2$) group with the acid ($-\text{COOH}$) groups of proteins. The formula for the ethyl ester of amino acetic acid is



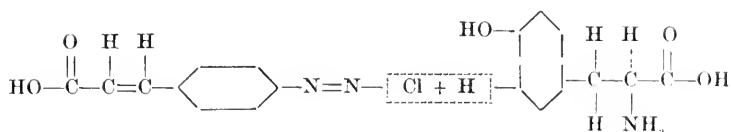
REMOVAL OF BASIC PROPERTIES OF AN AMINO ACID.—On the other hand, if the same amino acid (amino acetic acid) is acetylated, a compound is formed in which one of the hydrogens of the basic ($-\text{NH}_2$) group is replaced by the acetyl group, the formula for the resulting compound being



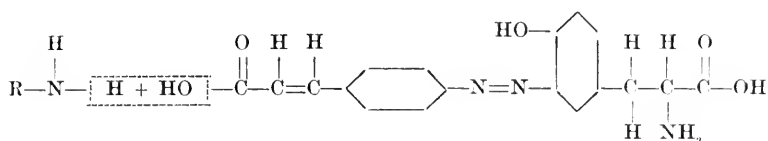
In this case the basic property is lost with a simultaneous increase in acid property so that the latter becomes dominant and union through replacement of the ionizable hydrogen to basic groups of other amino acids may occur and thus a new compound be formed through union with the salt-forming groups.

OTHER WAYS OF SALT FORMATION.—Another way in which salts may be formed may be illustrated as follows:

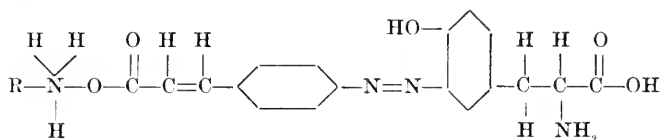
If one diazotizes p-aminocinnamic acid by means of nitrous and hydrochloric acids, there is obtained diazotized p-aminocinnamic acid which will couple with the nucleus of tyrosine as follows:



Now this product may unite with the $-\text{NH}_2$ group of another amino acid either by replacement of a hydrogen through loss of water to form an acid amide grouping as shown,



or by simple addition to form a substituted ammonium salt as shown by the formula

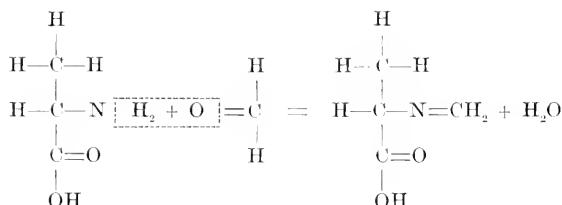


Effect of Esterification and Methylation of Protein.—Landsteiner (1917, 1918) brought about esterification of protein by use of alcoholic acid solution, methylation by use of diazomethane and acetylation by means of acid anhydrides or acid chlorides.

CROSS REACTIONS.—Proteins thus altered showed marked antigenic specificity depending upon the hapten introduced. Thus methylated horse protein reacted with immune sera for methylated proteins from other species of animals as well as with methylated plant proteins. This indicated that the hapten influenced the specificity of the protein-hapten complex. In the course of his studies he found that among the proteins coupled with diazonium derivatives different phenomena were noted.

SPECIFIC REACTIONS.—When he used them as antigens, those containing aniline, p-amino-azobenzenesulphonic acid, ortho-, meta-, and para-aminocinnamic acid and aminoazobenzenedisulphonic acid stimulated the production of specific antibodies reacting only with the homologous antigens, while others showed a broader sphere of activity.

Formalized Rabbit Serum as an Antigen.—Landsteiner and Lampl (1917) also showed that formalized rabbit serum used as antigen would stimulate antibodies when injected into rabbits that reacted only with formalized rabbit serum and not with formaldehyde-treated proteins of other species. This would indicate, as Wells (1929) has pointed out, that “the occupation of an amido group, e.g., lysine or alanine, by the methylene radical is almost devoid of any effect on specificity, and that a marked chemical change can take place in a protein without noticeable effect on the structural or species specificity.” When formaldehyde is added to a protein, it couples with the nitrogen of the amino ($-\text{NH}_2$) group with the splitting off of water, as may be seen in the following reaction of alanine and formaldehyde:



This condensation removes the basic properties of the amino acid and permits the acid groups to become dominant. This is the basis of Sörensen's “formol” titration method for amino acids.

Recent Work on Iodized Antigens.—Wormall (1930) and Jacobs (1932) have recently carried out extensive studies on iodinated sera. The latter prepared antigens, i.e., iodized serum proteins, in dilute ammonium hydroxide solution and also without acid or alkali and compared them immunologically. Jacobs also investigated the loss of original specificity as a result of iodine entering the protein as well as the specificity of the antibodies for iodized antigens and ascertained the amount of iodine necessary to alter the antigen. He also confirmed the observations of Wormall (1930) that diiodotyrosine suppressed the formation of specific precipitates that would ordinarily result when iodinated protein and its antiserum are mixed. This is another example of suppression phenomena frequently described by Landsteiner. Jacobs* summarizes the results of his work as follows:

*Jacobs, J.: J. Immunol. 23: 361, 1932.

(1) "When iodine is added to animal sera (antigen) without the presence of acid or alkali, substances are formed which precipitate with antisera prepared from iodinated sera.

(2) "This takes place in the presence of aqueous solutions as dilute as N/32.

(3) "Wormall's observation that diiodotyrosine, but not potassium iodide, inhibits precipitation specifically in systems of iodinated proteins and their antisera was confirmed."

MINIMUM AMOUNT OF IODINE TO CHANGE ANTIGENIC PROPERTY.—To ascertain the minimum amount of iodine necessary to alter a protein antigenically, he added 2.0 c.c. of iodine solutions corresponding to iodine concentrations of N/8, N/16, N/32, and N/64 to 1.0 c.c. of normal horse serum and the mixtures were allowed to stand at room temperature for fifteen minutes. Jacobs says that "a minimum amount of acetic acid was added and the precipitates were centrifuged, taken up in 10 c.c. of saline with the aid of just enough dilute carbonate to adjust the pH to approximately 7.5, and filtered through a Berkefeld V candle until clear." In the tests he used dilutions of this solution corresponding to 1:10, 1:50 and 1:250. "The supernatants were precipitated with three-fourths saturated ammonium sulphate, taken up in 10 c.c. of saline, pH adjusted to 7.5 and passed through a Berkefeld filter. Dilutions were made up comparable to those of the first precipitate. Both precipitates and supernatants were examined immunologically for the presence of normal horse serum and iodinated precipitinogen." He found that it required at least N/32 iodine to alter the antigen immunologically. In these altered antigens there remained unaltered horse serum. He observed a diminution of unaltered antigen as more iodine was added until only a trace was left in the preparations containing the maximum amount of iodine.

SPECIFICITY FOR IODIZED PROTEINS.—Like Obermayer and Pick (1906), Wormall (1932), Landsteiner and others, he found that the immune serum for iodized horse serum reacted with other iodized antigens.

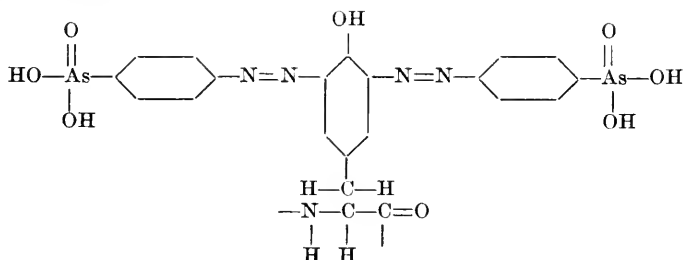
Group Reactions Due to Different Haptens in the Same Molecule.—Hooker and Boyd (1933) have coupled diazotized arsanilic acid to egg white and gelatin, respectively, and found them both to be antigenic despite the fact that gelatin is not antigenic. The

antiserum for each produced the maximum amount of precipitate with its homologous antigen, but the anti-egg-white-diazotized-arsanilic-acid protein gave weaker reactions with diazotized-arsanilic-acid-gelatin antigen and conversely the latter antiserum gave weaker reactions with the former.

EGG ALBUMEN CONTAINS TYROSINE AND HISTIDINE. GELATIN CONTAINS HISTIDINE.—It occurred to them that egg white contained both tyrosine and histidine, while gelatin contained histidine but no tyrosine. Hence egg white would have at least two amino acids linked to the arsanilic acid while gelatin would have only one; i.e., histidine. These are the two amino acids which couple with the diazonium compounds. When these antigens are injected one would expect two antihaptens to be developed for the diazotized arsanilic acid egg white and one for the diazotized arsanilic acid gelatin antigen. To determine whether this assumption was true they made use of the phenomenon of suppression of a specific antigen-antibody reaction by compounds chemically similar to the specific hapten on the antigen.

USE OF DIAZO DYES FOR SUPPRESSION OF SPECIFIC REACTION.—It will be remembered that Wormald showed that if he added diiodo-tyrosine to tubes containing antiserum for iodized protein (containing tyrosine) and its antigen, the specific precipitate was suppressed. In other words the addition of a hapten to a tube containing the hapten-protein antigen and its specific immune serum results in this case in the suppression of the precipitate. Hooker and Boyd prepared two dyes, one containing tyrosine-like diazoarsanilic groups and a second containing histidine-like imidazoldiazoarsanilic groups.

TYROSINEDIAZOARSANILIC GROUPS IN PROTEIN MOLECULE.—In the protein molecule the tyrosinediazoarsanilic groups are thought to be as follows:



They found that either dye will completely inhibit precipitation in either serum. The second, i.e., histidine-like, etc., "is relatively more efficient as an inhibitor for the antigelatin-diazo-arsanilic acid." They regard their results as indicating that "the injection of a single 'complete' protein coupled with diazotized arsanilic acid leads to the production of two different anti-haptens, one corresponding more closely to the tyrosinediazoarsanilic acid group, the other to the histidindiazoarsanilic acid group. In the case of the 'deficient' protein gelatin, the coupled protein seems to give rise to only one of these antihaptens."

These results indicate that more than one antibody may be produced by an antigen as suggested more recently by Landsteiner. All of the work on modified proteins supports Wells' (1929) contention that specificity may depend upon small groups in the molecule as well as other factors. For a brief summary of some of the more important points brought out in this chapter the student is referred to Chapter XX.

Antibodies to Strychnine.—Using Landsteiner's methods of producing new antigens Hooker and Boyd (1940) failed in an attempt to produce antibodies for morphine but were apparently successful in producing antibodies for strychnine. Precipitation with caseinazostrychnine was inhibited by the alkaloid and some of its derivatives but not by other substances, which like strychnine contain the indol nucleus. The sera they produced were too weak to neutralize the lethal effect of strychnine in mice.

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CHAPTER XIX

BACTERIAL ANTIGENS AND SPECIFICITY

Bacterial Antigens.—COMPLEXITY.—Landsteiner (1936), Wells (1929), Zinsser (1928, 1939), and others have called attention to the complexity of cellular or protoplasmic antigens as contrasted with isolated and purified proteins. While species specificity can, as a rule, be demonstrated for intact red blood cells by means of high-titered immune serum, one frequently encounters difficulty in determining bacterial species by agglutination, absorption, and complement fixation.

LIVING ATTENUATED ANTIGENS.—Interest in bacterial antigens began with the immunization experiments of Pasteur. He found that attenuated, living cultures were efficient vaccines for protective inoculation. Since that time the French school has continued to regard suspensions of living attenuated bacteria as the most desirable immunizing agents.

KILLED SUSPENSIONS AS ANTIGENS.—Widal (1896), Bordet, Gruber and Durham and others who initiated the serological identification of bacteria by agglutination, noted that dead suspensions of *E. typhosa* or *Vibrio comma* were quite satisfactory antigens. Later work by Felix and Pitt (1934) and others has shown the importance of labile antigenic factors in agglutination reactions.

EARLY RESEARCH ON ANTIGENS.—From Pick's (1908) review of the early literature it is evident that there were carried out extensive investigations of the effect of physical and chemical agents on the antigenic property of bacteria. He calls attention to the early use of heat-killed suspensions as well as to the use of phenol, formalin and other chemicals for the preservation of antigens or as germicidal agents.

SPECIES SPECIFICITY VS. IMMUNOLOGICAL SPECIFICITY.—Interest in the relationship between species specificity and immunological specificity developed early. In 1902 Castellani introduced the absorption technique. There soon developed among many bacteriologists a belief that by the use of agglutination and absorption

one could identify any bacterial species. For a number of species such as *E. typhosa*, *Vibrio comma* and several others, this is in a large measure true, although inagglutinable and atypically agglutinable strains of *E. typhosa* have been described by many workers. Their inagglutinability is apparently due to a labile "Vi" antigen. (Felix and Pitt, 1934.)

LIMITATION OF SEROLOGICAL METHODS.—While there is an increasing amount of evidence suggesting that each species possesses a species-specific antigenic fraction or component, yet this is not always demonstrable by agglutination or complement fixation using unaltered bacterial suspensions. In fact, the result of agglutination and absorption work would have one to believe that most species of bacteria are not represented by a species-specific antigen (in this case an agglutinin) but on the contrary by two or more antigenic types. It is only by special methods of investigation that one can frequently demonstrate species-specific antigenic substances. It may be that ultimately immunochemical studies will lead to a more accurate classification of bacteria.

Stevens (1923) concludes that not all strains of a specific organism can be recognized by agglutination.

SPECIES SHOWING ANTIGENIC HOMOGENEITY.—The results of immunological and biological investigations using agglutination and absorption and also complement fixation techniques indicate that bacterial antigens fall into three divisions. The first includes those species, each of which consists of one dominant antigenic type. Practically all strains can be identified by means of an immune serum prepared against any single strain.

SPECIES SHOWING A FEW SEROLOGICAL TYPES.—The second division is composed of species, each of which is represented by a small number of serological types and perhaps a heterogeneous one. With these it is possible to use a polyvalent immune serum for species identification and supplement this with other methods designed for the heterogeneous group of each.

SPECIES SHOWING ANTIGENIC HETEROGENEITY.—The third division consists of those species, each of which shows so much antigenic heterogeneity that agglutination and absorption or even complement fixation methods are not practical for species identification.

This will probably be best appreciated from a study of the following list of species belonging to each of the divisions mentioned above, together with reference to specific immunological research relative to each species.

Division I. Species which can be identified serologically by means of a high-titered monovalent, species-specific immune serum.

E. typhosa (Downs, 1925); *Mycobacterium tuberculosis* (Rice and Reed, 1932, Wilson, 1925); *Pfeifferella whitmorei* (Stanton and Fletcher); *Microspira comma* (Douglas, 1929); *Coccobacillus foetidus ozanea* (Topley and Wilson 1:309); *Pasteurella pestis* (Topley and Wilson 1:486); *H. pertussis* (Topley and Wilson 1:505); *Brucella abortus* (Walton, 1930, and Topley and Wilson 1:515); *Cl. chauvei* (Topley and Wilson 1:533); *Treponema pallidum* (Rice, 1932, also Topley and Wilson 1:570); *Staphylococcus aureus* (Julianelle, 1922, also Topley and Wilson 1:391).

Division II. Species, each represented by a limited number of distinct antigenic types, some subtypes and perhaps a heterogeneous group.

Streptococcus hemolyticus (Topley and Wilson 1:373); *Neisseria gonorrhoea* (Atkin, 1925, also Topley and Wilson 1:356); *Neisseria meningitidis* (Branham, 1932, also Topley and Wilson 1:341); *B. dysenteriae* (Topley and Wilson 1:458); *Friedlander bacillus* (Julianelle, 1926, also Topley and Wilson 1:464, 465, 469); *B. mallei* (Topley and Wilson 1:307); *Cl. tetani* (Topley and Wilson 1:557); *B. paratyphosus* B. (Jordan, 1923); *B. enteritidis* (Sherwood, Downs and McNaught, 1920).

Division III. Species which show such extreme antigenic heterogeneity that immune serum is of no value in species identification.

B. coli (Dulaney, 1928, Mackie, 1913, also Topley and Wilson 1:427); *B. proteus* (Topley and Wilson 1:408); *Rhizobium leguminosarum* (Topley and Wilson 1:315); *C. diphtheriae* (Topley and Wilson 1:287); Aerobic spore producers (Treece, 1920); *Cl. welchii* (Topley and Wilson 1:540); *Streptococcus viridans* (Hooker and Anderson, 1929); *Pseudomonas pyocyaneus* (Sherwood, Johnson and Radotinsky, 1926).

Lack of Standard Procedure.—It should be remembered that these three divisions are based upon agglutination, absorption and complement fixation work by different individuals using various techniques and cellular antigens prepared in different ways. While type-specific polysaccharides have been found in a number of species, not many have been studied extensively to ascertain what haptens and antigenic fractions they contain and whether one or more are species specific. In some of the studies reported, methods of extraction have been used that have apparently racemized or denatured the bacterial antigens. One could deal more intelli-

gently with bacterial antigens if more were known about their chemistry. This subject has been dealt with recently by Baumgartel (1928), Branham (1928) and Zinsser, Enders and Fothergill (1939). Some of the more recent literature has been reviewed by Heidelberger (1932, 1933) and Marrack (1938).

Errors Due to Presence of Extraneous Material in Antigens.—

In the preparation of bacterial antigens little attention has been paid to such factors although several have called attention to errors in immunological studies due to the fact that the bacterial antigens contained substances from the culture media that led to erroneous conclusions.

This possibility of error in immunological research is indicated by the observation of Sordelli and Mayer (1931) reported by Heidelberger,* "that a 1:400,000 solution of agar (gelose) is enough to yield precipitates with antityphoid and antianthrax sera." Heidelberger suggests that this may account for cross-reactions reported by many workers.

Early Work on Antigenic Fractions.—Perhaps one of the earliest studies on antigenic fractions of bacteria was that of Koch (1891, 1901) who found that extracts of the tubercle bacillus produced specific reactions when injected into tuberculous animals. He regarded the extracts as containing an active principle of protein nature which he called *tuberculin*.

SUBSEQUENT INVESTIGATIONS.—This has led to extensive investigations by Long and Seibert (1926), Johnson and Coghill (1926, 1931, 1935), Anderson (1927, 1931, 1933), Zinsser and Rice and Reed (1932), Dienes (1929, 1930) and Dienes and Freund (1926), as well as many others, on the chemical and antigenic components of the acid-fast group, especially the various strains of the tubercle bacillus.

PROPERTIES OF TUBERCULIN.—Seibert (1926, 1933, 1934) succeeded in crystallizing tuberculin and studying some of its properties. The crystals, according to Long (1928), are water-soluble, take a methylene blue stain, give the biuret and Millon tests and are heat coagulable. The properties of a new Purified Protein Derivative of Tuberculin prepared by Seibert (1932, 1934) are discussed in Chapter XXVI.

*Heidelberger: Ann. Rev. Biochem. 1: 655, 1932.

In the past all attempts to employ tuberculin in antigen-antibody reactions have failed. Seibert (1935) reports that the precipitin reaction between tubercle protein and its homologous antiserum is inhibited by her tuberculin preparation SOTT having a molecular weight of 3800. By means of the ultracentrifuge and electrophoresis Seibert, Pedersen and Tiselius* (1938) have studied the constituents of tuberculin. They have isolated a protein with a molecular weight of 32,000 from culture filtrates. This protein, when injected into tuberculous guinea pigs, caused death. They isolated substances of lower molecular weight (9,000-16,000), from old tuberculin, that were active in producing skin reactions. According to Chase and Landsteiner (1939), Maschmann (1937) has reported success in separating tuberculin into two fractions, one (resistant to proteolytic enzymes) that gives systemic and the other (destroyed by trypsin) that gives positive skin reactions.

Specific Substances in Bacteria.—EARLY DISCOVERIES.—In 1921 Zinsser obtained a specific substance from tubercle bacilli. It was obtained by alkaline extraction of ground tubercle bacilli, and was heat stable, alcohol-insoluble, and gave positive precipitin and complement fixation tests with specific immune serum.

A gumlike substance that reacted with serum of animals immunized with tubercle bacilli was isolated by Laidlaw and Dudley (1925). Tubercle bacillus polysaccharides were isolated by Heidelberger and Menzel (1932) and Seibert, Pedersen and Tiselius (1938). Heidelberger and Menzel (1938) have continued their studies of tubercle bacillus polysaccharides and have been able to isolate two which are immunologically active; one is low and the other is high in pentose. They "are apparently built up chiefly of d-arabinose and d-mannose units in varying proportions."

Pick (1912) had isolated similar soluble specific substances from *E. typhosa* and Dochez and Avery (1917) had found in the urine and blood of pneumococcus pneumonia patients a substance that gave specific reactions with pneumococcus immune serum. In 1923, Zinsser and Parker found similar substances in pneumococci, staphylococci, influenza and typhoid bacilli. Heidelberger and Avery have shown the soluble specific substances of pneumococci to be polysaccharides and type-specific. These and other important reacting carbohydrates and proteins isolated from a number of other bacteria will be discussed briefly later in this chapter.

*Seibert, F. B., Pedersen, K. O., and Tiselius, A.: J. Exper. Med. 68: 413, 1933.

Antigenic Comparisons of Acid-Fast Bacteria.—Lewis and Seibert* (1931, 1933) have made extensive antigenic comparisons of different strains of the tubercle bacillus and the timothy bacillus. They found definite antigenic relationships and also differences between the human, bovine and avian strains but only slight relationship between any of these and the timothy bacillus.

It has been shown that tubercle bacilli contain lipoids, proteins and carbohydrates. The question as to the immunological importance of each of these has led to much controversy. All three types of substances have been reported as giving serological reactions.

They conclude that, "The sensitized animal body is able to make a quantitative distinction between the proteins from different types of acid-fast bacilli, but is apparently unable to differentiate the proteins from different strains of the same type, such as between virulent and avirulent organisms."

LIPIDS.—In regard to the lipoids, Wells (1929) calls attention to the possibility that perhaps many of the lipid preparations that have been studied contain a trace of protein. He considers that alcohol-soluble proteins may be present in bacterial extracts and that their presence might account for the results of Klopstock and Witebsky (1927) who found that alcoholic extracts of bacteria were antigenic and Pinner (1927, 1928) who noted an increase in the antigenic property of alcoholic extracts of tubercle bacilli as the protein content was diminished by purification.

RELATIVE IMPORTANCE OF LIPIDS, PROTEINS AND CARBOHYDRATES.—There seems to be fairly good evidence indicating that the lipid fraction of the tubercle bacilli is similar for all strains and that it does not determine serological specificity. On the other hand, the protein fraction, containing carbohydrate configurations, is quite specific. Long (1930) considers that the polysaccharides of the tubercle bacillus are immunologically specific as shown by Laidlaw and Dudley (1926), Mueller (1926) and Zinsser and Tamiya (1925). His conclusions are apparently confirmed by the later work of Seibert, Pedersen and Tiselius (1938) and Heidelberger and Menzel (1938).

*Lewis, J. H., and Seibert, F. B.: J. Immunol. 20: 201, 1931.

Anderson (1932) and also Smedley-MacLean (1932) have reviewed the work of Anderson (1930, 1931), Sabin and Doan (1927) and of Doan (1929) on the fatty acids and phosphatids isolated by Anderson from the members of the acid-fast group. Anderson (1940) has extended his work on lipids of the tubercle bacillus. He reports that two polysaccharide fractions isolated from two preparations of phosphatids gave different cleavage products.

TUBERCULO-PHOSPHATIDS AND FATTY ACIDS.—The tuberculo-phosphatids differ from phosphatids obtained from other sources in their phosphorus and nitrogen content. According to Anderson they also contain a large percentage of what appears to be a new type of polysaccharide. On hydrolysis the latter yields mainly mannose and inosite. Pangborn and Anderson (1933) and Anderson and Newman (1933) have also reported on the isolation of trehalose from the timothy and tubercle bacilli, respectively.

Immuno-Chemical Studies of the Pneumococcus.—Another species of bacteria that has been both extensively and intensively studied from the standpoint of immunochemistry is the pneumococcus. Attention has already been called to the early discovery of Dochez and Avery (1917), Zinsser (1921), Zinsser and Parker (1923), of soluble specific substances from pneumococci that are type specific in that they give specific precipitates or positive complement fixation when mixed with their respective immune sera. These substances have been carefully investigated by Heidelberger, Goebel, and Avery* since 1923. Their results may be briefly summarized as follows:

PROPERTIES OF TYPE SPECIFIC POLYSACCHARIDES.—In the beginning they found that all three types of specific soluble substances are polysaccharides quite dissimilar chemically as well as immunologically. Unfortunately they employed methods at first which deacetylated the natural soluble specific substance. In view of the observations of Schiemann and Casper (1927), Saito and Ulrich (1928), Enders (1930) and Wadsworth and Brown (1931) that pneumococci contain an antigenic polysaccharide, Avery and

*Heidelberger, Goebel, and Avery: J. Exper. Med. 38: 73, 1923.

Goebel (1933) reinvestigated the type specific polysaccharide of Type I and came to the following conclusions:

1. The soluble specific substance of Type I is an acetyl polysaccharide.

2. The methods they employed in 1923 in isolating Type I SSS resulted in deacetylating the natural acetyl polysaccharide.

3. The acetyl polysaccharide is antigenic in that the injection of it into mice leads to the development of an immunity to Type I pneumococcus but not to the formation of precipitins. Zinsser and Bayne-Jones (1934) speak of this as a functional immunity.

4. The acetyl polysaccharide is quite soluble in water.

5. It shows a specific optical rotation in aqueous solution of +270.

6. It has a nitrogen content of 4.8 per cent. When treated with nitrous acid in the cold, about 45 per cent of the nitrogen is liberated in the amino form.

7. It yields reducing substances when hydrolyzed with dilute mineral acids. When the reducing substance appears, the serological specificity is lost.

8. The type-specific polysaccharide is also soluble in 80 per cent acetic acid.

9. Dilute aqueous solutions (0.5 per cent) are precipitated by silver nitrate, neutral and basic lead acetate and phosphotungstic acid. It is incompletely precipitated by barium hydroxide. It is precipitated by tannic acid but not by uranyl nitrate.

10. The ninhydrin, biuret, picric acid and sulphosalicylic acid tests are negative.

11. Neither phosphorus nor sulphur was detectable in highly purified preparations of the acetyl polysaccharide.

Marraek (1938) describes Type I polysaccharide as containing a basic group and a trisaccharide with two uronic acid molecules and amino sugar.

Brown (1939) has summarized in tabular form the chemical data on the soluble specific substances of Types I to XXXII pneumococci. Apparently they all contain dextrose although only Types V, XI, and XXXII will reduce Fehling's solution before hydrolysis. Types II, III and VIII are thought to be composed of dextrose and aldobionic acid in a carbohydrate chain. According to Brown's data uronic acid was present in Types I, II, III,

VIII, IX, XII, XXII, XXV, and XXVI. She reports amino sugar (after hydrolysis) in Types I, IV, V, VII, IX, X, XII, XIII, XIV, XV, XVI, XIX, XX, XXI, XXIV, XXV, XXVII, XXIX, XXX, and XXXI. Types I, IV, V, XII and XXV contain the largest amount of nitrogen (about 5%). Type I alone has an appreciable amount of amino nitrogen (2%). Edwards, Hoagland and Thompson say that in the case of the specific polysaccharides of Types II, III and VIII the difference appears to be principally one of stereochemistry. These polysaccharides are haptens (Type I is definitely antigenic) coupled to protein common to all pneumococci. The combination forms a complete pneumococcus antigen that is type specific. The type specificity is due to the polysaccharide hapten.

Finland and Curnen (1938) report an interesting discovery concerning the specific carbohydrate of pneumococcus XIV. They observed that horse immune serum for this type of pneumococcus contained agglutinins for human red cells. According to Hoagland, Beeson and Goebel (1938) this substance resembles to a great extent the group "A" substance from pig stomach.

DIFFERENCE BETWEEN IMMUNE SERUM FROM HORSES AND RABBITS.—Heidelberger and Kendall (1933) have also found that partial hydrolytic products of Type III specific polysaccharide can be quantitatively freed from unhydrolyzed polysaccharide. They find that the fractions yield specific precipitates with Type III antipneumococcus serum obtained from horses but fail to give a precipitate with similar immune serum obtained from rabbits. In their opinion Felton's studies on antibodies for the pneumococcus may offer an explanation of this. Felton found that pneumococcus antibodies obtained from horse serum are precipitated with the water-insoluble fraction of the serum globulin* whereas rabbit antipneumococcus sera yield no precipitate on dilution.

POSSIBLE EXPLANATION OF INHIBITION PHENOMENON.—They say that "The failure of the hapten fraction to form insoluble compounds with rabbit antibody may be connected with the greater tendency of rabbit globulin to remain in solution." They also suggest that this may explain the inhibition phenomenon of Landsteiner and van der Scheer.

*J. Immunol. 21: 341, 1931.

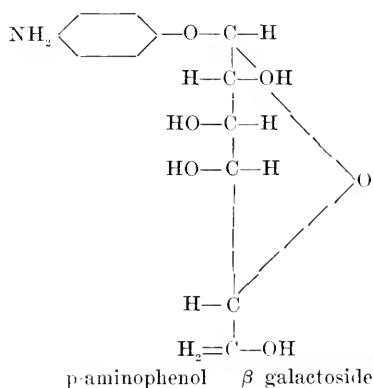
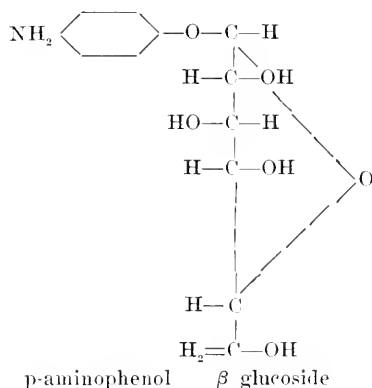
Landsteiner (1936, p. 119) suggests that the inhibition phenomenon may be due to an excess of antigen resulting in the formation of soluble compounds containing a larger proportion of antigen, in comparison to antibody, than there is in precipitates.

THE "C" SPECIES-SPECIFIC BUT NOT TYPE-SPECIFIC SUBSTANCE OF TILLET AND FRANCIS (1930).—This substance has been studied by Tillett, Goebel and Avery (1930). They find that it is a non-protein fraction distinct from the type-specific polysaccharide. The evidence seems to indicate that it is a "nitrogenous polysaccharide analogous in chemical behavior but not in serological reactivity to the Type I soluble-specific substance." Since it passes through collodion membranes with ease and quite readily through parchment membranes, Tillett, Goebel and Avery conclude that the molecule is smaller than that of the SSS previously described. The "C" fraction is apparently common to all pneumococci. It is evidently not related to virulence since it is present in the avirulent "R" forms as well as the virulent "S" forms. Heidelberger and Kendall have found the "C" substance in Type IV pneumococci and have studied its chemical properties. Its rotation is given as $+40^\circ$ and its nitrogen content as 6.1 per cent. They also found approximately 0.9 per cent of amino nitrogen. It differs from the pneumococcus type-specific substance in containing phosphorus (4.0 per cent) (Heidelberger, 1932). The somatic substance is said to include skin irritating nucleic acids and nucleoproteins.

CARBOHYDRATE FRACTIONS ADSORBED ON CARBON PARTICLES.—While all previous work has shown that the soluble-specific substances (SSS) of Types II and III are nonantigenic, yet Zozaya and Clark have recently (1933) reported active immunization of mice with the polysaccharides (SSS) of pneumococcus Types I, II, and III. They used polysaccharides adsorbed on carbon particles and also various dilutions of unadsorbed material. They claim that the dilution factor is important. Their work needs further confirmation. It should also be noted that Wadsworth and Brown call attention to the importance of dosage in the demonstration of the antigenic nature of the carbohydrate fraction described by them.

Synthetic Carbohydrate Haptens.—In the course of their investigations of the rôle polysaccharides play in determining immunological specificity, Goebel and Avery (1929) synthesized two

optically isomeric polysaccharides, e.g., p-aminophenol-glucoside and the corresponding galactoside. They diazotized these, using nitrous acid and hydrochloric acid in the cold as discussed in Chapter XVIII. These diazonium derivatives were readily coupled onto the proteins (horse serum globulin) in the presence of N/100 hydroxide. They then studied and compared their antigenic properties. The structural formulae for the two isomeric glucosides used as haptens in these experiments are given by Avery and Goebel (1929) as follows:



The specificity of the conjugated hapten proteins was checked further by linking the same diazotized glucosides not only to horse serum globulins but also to egg albumen. Avery and Goebel* summarize their results as follows:

1. When two chemically different carbohydrate derivatives are bound to the same protein, the newly formed antigens exhibit distinct immunological specificity.
2. When the same carbohydrate radical is conjugated with two chemically different and serologically distinct proteins both of the sugar-proteins thus formed acquire a common serological specificity.
3. The newly acquired specificity of the artificially prepared sugar-proteins is determined by the chemical constitution of the carbohydrate radical attached to the protein molecules. Simple differences in the molecular configuration of the two isomers, glu-

*Avery and Goebel: J. Exper. Med. 50: 533, 1929.

cose and galactose, suffice to orientate protein specificity when the corresponding glucosides of the two sugars are coupled to the same protein.

4. The unconjugated glucosides, although themselves not precipitable in immune serum, inhibit the reaction between the homologous sugar-protein and its specific antibody. The inhibition test is specific.

5. The sugar derivatives unattached to protein exhibit the properties of carbohydrate haptens; they are nonantigenic but specifically reactive, as shown by inhibition tests.

Following this work, Avery and Goebel (1931) coupled the nitrogen-free polysaccharide of Type III pneumococci onto horse serum globulin after preparing p-amino and p-nitromonobenzyl ethers of the specific polysaccharide. They produced active immunity and type-specific antibodies in rabbits for Type III pneumococcus by injecting the polysaccharide horse serum globulin antigenic complex. They were also able to protect mice specifically by immunization with the same.

Hotchkiss and Goebel (1937) and Goebel (1938, 1939) have extended the work on synthetic conjugated antigens. The former synthesized azoproteins containing glucuronic acid and galacturonic acid respectively. The galacturonic acid azoprotein gave positive precipitin reactions with Type I anti-pneumococcus serum. In 1939 Goebel produced an antigen containing cellobiuronic acid that produced active immunity in rabbits to virulent Type III pneumococci. Serum from these immunized rabbits conferred passive immunity to mice for Types II, III, and VIII pneumococci. The serum also gave positive Neufeld quellung reactions.

Friedländer's *Bacillus*.—In 1925 Heidelberger, Goebel and Avery became interested in the capsular substance of a strain of the Friedländer's bacillus. Earlier (1921) Toenniessen had isolated from the capsular substance of a strain of this organism, a snow-white nonreducing substance that was a polysaccharide substantially free of nitrogen. Upon hydrolysis it yielded reducing sugars. Toenniessen found that these yielded an osazone which he regarded as that of galactose. His work was confirmed by Kramár (1922). The substance was not studied immunologically

until 1924-25 when Mueller, Smith and Litareczek reported that the polysaccharide thus obtained caused specific precipitation of homologous immune serum.

Heidelberger, Goebel and Avery (1925) found that the polysaccharide from strain E (Type B of Julianelle) is chemically and immunologically similar to but not identical with the soluble specific substance of Type II pneumococcus. They found that immune serum for strain E of Friedländer's bacillus agglutinates both the homologous organism and also Type II pneumococcus and conversely immune serum for the latter agglutinates both organisms. Similar results were obtained using the respective polysaccharides and corresponding immune sera. When absorption experiments were carried out, however, it was found that when Friedländer (strain E) immune serum was absorbed with Type II pneumococcus, the agglutinins for the pneumococcus were removed, but there remained agglutinins for the homologous organisms; and likewise when pneumococcus Type II serum was absorbed with Friedländer's bacillus (strain E) the agglutinins for the latter were removed, but there remained agglutinins for Type II pneumococcus. When either immune serum was absorbed with its homologous organism, agglutinins for both were removed. Similar results were obtained using the respective polysaccharides and immune sera.

It is interesting to note that Julianelle (1926) found immunological types to exist among forty strains of Friedländer's bacillus studied. These strains fall into three specific types which he designates as A, B, and C and a heterogeneous one which he has named Type X. The strain whose capsular substance is similar to that of pneumococcus Type II corresponds to Type B of Julianelle.

The soluble specific substances of Types A and C have been studied extensively by Goebel (1927) and Goebel and Avery (1927). The former investigated the hydrolytic products of Type A polysaccharide and found that it yielded on hydrolysis an aldobionic acid, glucose, and a second acid not identified. These three compounds were found to be present in the ratio of 1:1:1. Goebel found that the new aldobionic acid consists of a molecule of glucuronic acid linked to a molecule of glucose through its reducing group. It is *isomeric* with an acid derived from the soluble

specific substance of Type III pneumococcus. The polysaccharide appears to be a condensate of 2 molecules of aldobionic acid and 1 molecule of glucose.

The Type A substance yields specific immunological reactions with the homologous type immune serum, but does not give cross-reactions with any one of the three type specific pneumococcus immune sera. The failure to react with pneumococcus Type III serum, considering its chemical similarity to Type III polysaccharide, is apparently due to isomeric configuration.

Goebel and Avery (1927) found that Friedländer's Type C soluble specific substance is also a nitrogen-free polysaccharide and that it is chemically similar to the one obtained from Type B. Two distinct differences were noted; they show no cross-relationship immunologically; they differ in their solubility in water and in their behavior during purification. Pure Type B is difficultly soluble, while the Type C substance is readily soluble in water. The former is readily precipitated by alcohol in the presence of hydrochloric acid, whereas the latter precipitates completely only after standing for one hour at 0° C. Avery and Goebel* (1927) think that the immunological dissimilarity observed in these two substances that are physically and chemically so much alike is probably due to "slight differences in the intramolecular linkage of sugar to sugar, or of sugar to sugar acid."

***Escherichia coli*.**—Stuart, Baker, Zimmerman, Brown and Stone† (1940) report the literature along with their own experimental studies of the relationship of coliform bacteria. They say that Magheru (1937) found the entire "O" antigen of *Escherichia coli* is composed of a sugar-lipoid complex and that the complete antigen was not contained in all of the variants. Precipitin studies indicate many serological varieties for *Escherichia coli*. Torrey (1938) has found a virulence factor associated with the SSS element but not identical with it.

***E. typhosa*.**—According to Topley et al. (1937) two carbohydrate-lipid complexes corresponding to the "O" and "Vi" antigens of *E. typhosa* have been isolated. These two substances differ in their carbohydrate content and in a few other ways.

*Avery and Goebel: J. Exper. Med. 46: 601, 1927.

†Stuart, Baker, Zimmerman, Brown, and Stone: J. Bact. 40: 101, 1940.

Somewhat analogous findings have been reported, according to Chase and Landsteiner, for *S. paratyphi C* and certain *Pasteurellas*.

Shigella dysenteriae.—The antigenic complex of Shiga has been studied by Morgan (1936, 1937, 1938). He reports that it stimulates the production of both antibacterial and heterophile antibodies and is thought to be the endotoxin of Shiga. Upon fractionation he obtained in addition to the specific polysaccharide, lipoidal and other material. The polysaccharide contains 1.6 per cent nitrogen and yields 98 per cent reducing sugar on hydrolysis. The antigenic substance then appears to be a carbohydrate-lipid complex.

V. cholerae.—Studies of cholera vibrios has resulted in what appears to be the establishment of six groups according to Mitra. Two types were distinguished by differences in globulin fractions. Landsteiner and Chase (1939) say that when these results are considered together with three sorts of specific carbohydrates that have been isolated it "permits of the establishment of six groups of vibrios."

White (1937) studied the O receptor complex of strains of *V. cholera* found in India. He found the O receptors to be antigenic and located in the specific polysaccharide. He does not assume that the antigenic factors causing multivalence represent so many different substances. Instead he thinks of the multivalence as due to the presence of individual receptor substances in the polysaccharide molecules and to combinations of these functioning as complex receptors. He postulates the existence of three primary receptor groups in the cholera polysaccharide molecule.

H. influenzae.—It was formerly thought that *H. influenzae* organisms constituted a heterogenous group. Zinsser and Bayne-Jones (1939) are of the opinion that the smooth *H. influenzae* represent a homogenous group. It is only after dissociation into "R" forms that they become serologically heterogeneous. Pittman (1931) found that the "S" forms produced a soluble specific substance. Dingle and Fothergill have isolated this substance and identified it as a carbohydrate. Serological tests with this substance gave positive precipitation in high titer with antisera obtained from both horses and rabbits. While numerous cross re-

actions were encountered with the immune serum from horses, the rabbit immune serum was specific.

H. pertussis.—While a number of types of *H. pertussis* have been reported in the literature it appears that the types reported represented serological differences between mutation forms described by Shibley and Hoelscher (1934). Leslie and Gardner (1931) described four dissociation phases of *H. pertussis*. They termed them Phases I, II, III and IV. It appears that the Phase I is the hemolytic smooth form that is found in whooping cough. It will be recalled that Sauer's vaccine is made from hemolytic, capsulated, virulent Phase I of *H. pertussis*.

Brucella abortus.—There has been some controversy over the nature of the specific substance in *Brucella abortus*. Hershey, Huddleson and Pennell (1935) reported the specific reactions described by others as due to a noncarbohydrate substance. In later work they have found small amounts of carbohydrate in the antigenic material. Libby and Joyner (1941) report isolating an antigenic carbohydrate from all three strains of *Brucella*. Cutaneous tests with this carbohydrate in infected or sensitized subjects results in an immediate allergic reaction. There is no delayed reaction as in the tuberculin test.

Hemolytic Streptococci.—By means of immune sera obtained from rabbits, Griffith has been able to divide hemolytic streptococci from scarlet fever cases into four types and a heterologous group. Satisfactory typing of streptococci has not been as adequately solved by agglutination as it has by precipitation methods using extracts of streptococci. Lancefield by means of the precipitin reaction has confirmed and extended Griffith's work on the division of hemolytic streptococci into groups and types. She has described groups "A" to "G," the group division depending on the occurrence of a specific carbohydrate (C) for each group. Type specificity in the case of group "A" depends on the presence of an antigenic protein "M." In the case of the other groups, type specificity rests on the presence of polysaccharides designated as (S). These type specific substances are present in the mucoid and Matt type colonies of group "A" and in the smooth colonies of the other groups but are lacking in the glossy "A" colonies and in the rough colonies of other groups.

There is some correlation of type specific substance with virulence, but a strain of "A" group may lose its virulence and still retain the Matt type of colony.

This work has assumed great importance because of the consistency with which group "A" streptococci are associated with human infection.

Recently another type specific antigen designated as "T" has been found in group A streptococci in both Matt and glossy strains. It has not been defined chemically but it is responsible for type specific agglutination. Anti "T" serum exerts no protective effect in mice. The following antigens have been described by Lancefield:

Group specific carbohydrates	(C)
Type specific protein for group "A"	(M)
Type specific substances	(T)
Type specific carbohydrates for groups other than "A"	(S)

To obtain extracts for use in the precipitin work Lancefield extracts the streptococci with hot (boiling) N/20 HCl to destroy a Group factor "P" which overlaps with other organisms like pneumococci. She separates the type specific "M" substance from the Group specific (C) carbohydrate by neutralizing the acid extract and precipitating out the "M" substance with alcohol. The Group specific polysaccharide "C" remains in the supernatant fluid. For detailed description of the technique involved the student is referred to Lancefield's original paper. They might also be interested in a modification of Lancefield's method reported by Brown* (1938) and in a new method suggested by Fuller (1938).

Other Bacterial Specific Substances.—The specific substance of meningococcus Type I is reported by Scherp and Rake (1935) as sodium salt of a polysaccharide acid containing firmly bound phosphoric acid. Julianelle and Wieghard (1935) have described two type specific polysaccharides for staphylococci. Sievers and Zetterberg report that spontaneous agglutination interfered with their experimental study of aerobic spore forming bacteria. They obtained results with autolysates which suggests the existence of a

*Brown, J. H.: J. A. M. A. 111: 310, 1938.

specific antigenic structure in the different types. Ivánovics and Bruckner (1937) (1938) and Tomesik and Ivánovics (1938) have described a new type of hapten which they have isolated from the capsular substance of two aerobic spore producers, *B. anthracis* and *B. mesentericus*. It is a polypeptide of high molecular weight containing only one amino acid.

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CHAPTER XX

RECAPITULATION OF CHAPTERS ON SPECIFICITY

Examples of Specificity in Nature.—In the three preceding chapters the subject of specificity is discussed rather extensively. It is illustrated by the observations of Loeb and others made during fertilization experiments, by the physician in skin grafting and blood typing, by the immunologist who is interested in the acute infectious diseases and in biological relationships, by the physiologist in his studies of hormones, enzymes, and various physiological phenomena, by the pharmacologist in his experiments dealing with the nature and pharmacological action of drugs and by the geneticist in his investigation of inheritance. In fact, it is evident that the phenomenon of specificity is a common one in Nature.

It is important in medicine because all of our serological tests and many therapeutic procedures must be reasonably specific to be of value.

Antigens.—In earlier chapters attention is called to the fact that the injection of some substances into the animal body may lead to the production of specific antibodies, while the injection of other substances never leads to the production of antibodies. Specific antibody stimulators are called *antigens*. Their properties are reviewed in these chapters. They are all colloids and aside from a few apparent exceptions, belong to the group of organic compounds called proteins. The antigenic properties of various proteins are compared with their chemical structure and a number of important facts recorded:

PROPERTIES OF PROTEIN ANTIGENS.—

(a) Proteins are made up of amino acids linked together through their amino and carboxyl groups. They differ from each other according to the number, kind, and arrangement of the amino acid components. Since all proteins give the Molisch reaction, it is possible that each contains a carbohydrate radical. There is some evidence that globulins contain a lipid. If carbohydrate or lipid is present, it will increase the number of possible combinations of

protein elements and thus add to the number of different proteins. It has been estimated that with the twenty amino acids it is possible to build 2,432,902,008,176,640,000 different kinds of proteins. It appears from the work of Seibert and others that the size of the molecular aggregate forming the particles of colloid is another important factor determining the antigenicity of a substance.

(b) Only those proteins containing certain of the aromatic amino acids are antigenic.

(c) For a protein to retain its antigenic property, the optical activity of its amino acids must be unimpaired.

(d) Antigens must be soluble in the body fluids.

(e) It has been determined by comparing the chemical structure and antigenic property of a large number of plant and animal proteins that immunological specificity is dependent upon the chemical constitution of the antigen.

SPECIES-SPECIFICITY AND TYPE-SPECIFICITY.—Certain proteins such as those in the crystalline lens of the eye are common to all mammals and are therefore not species-specific. The same can be said of casein, thyroglobulin, testicular protein and blood fibrinogen. Some proteins are shared by a few species only, as, for example, gliadin is present in wheat and rye, while other proteins such as the serum globulins, albumins and tissue fibrinogens are species-specific. Thus it will be seen that the body contains many chemically and therefore antigenically different proteins, some of which are species-specific and others are not.

DIFFERENCES WITHIN A SPECIES.—Attention is called also to biochemical and antigenic differences within a species. There are four antigenic types of human red blood cells, over fifty types of pneumococci, several types of tetanus bacillus, etc. When one inspects these cellular antigens in which each species is represented by several antigenic types, he discovers that all types of any one species have a species-specific antigenic protein in common, and that each type is composed of a different hapten associated with the species-specific protein. Thus, all pneumococci have a species-specific pneumococcus protein in common, but Type I has, in addition, a hapten (polysaccharide) that distinguishes it from other pneumococci; likewise Types II, III, etc., each possesses distinguishing haptens (polysaccharides), all chemically different from each other. In the case of the four types of human red cells, all

possess a species-specific human protein in common, but there are two haptens, "A" and "B," which make possible these four types. One type is represented by the species-specific protein not associated with either hapten, this is type "O." A second type exists because of the presence of hapten "A," a third because of hapten "B," and a fourth has both "A" and "B" associated with the species-specific protein. It is interesting to note that haptens may be carbohydrate-lipoid complexes, as, for example, the "A" and "B" factors of red cells or the hapten fraction of Forsmann's antigen or *perhaps* just lipoids as suggested by Eagle for the antigen used in the Wassermann reaction. They may be pure carbohydrates such as one finds as partial antigens representing many bacterial types within a species as well as characterizing some species; or the hapten may be some chemical group such as tartaric acid, etc., attached to a protein.

HAPTEN MAY DOMINATE A SEROLOGICAL REACTION.—Most haptens are incapable of stimulating antibody formation when separated from the protein fraction and injected into the animals. Antibodies formed as a result of injecting the hapten-protein complex can react with either the hapten alone or the hapten-protein combination. It seems that the hapten dominates the picture to the extent that one may not observe the presence of antibodies for the species-specific protein part of the antigen in immune serum which theoretically should contain them.

Suppression Phenomenon of Landsteiner.—In connection with haptens the "suppression phenomenon" of Landsteiner is of interest. This is illustrated by the work of Wormall. He iodized a protein that contained tyrosine and then prepared a good precipitating antiserum for the iodized protein. He found that when he added diiodotyrosine to tubes containing the iodized antigen and its antiserum, the specific precipitate did not form, it was suppressed.

ARSANILIC ACID COUPLED TO HISTIDINE AND TYROSINE BY HOOKER AND BOYD.—By means of this phenomenon Hooker and Boyd were able to prove that the arsanilic acid which they linked to gelatin couples to histidine, and when linked to egg white it couples with both tyrosine and histidine. They prepared good precipitating antisera for the arsanilic acid gelatin and egg white antigens respectively. They then synthesized tyrosine-like arsanilic acid and

histidine-like arsanilic acid haptens and found that only the latter would suppress the precipitate when the arsanilic-acid-gelatin antigen was mixed with its antiserum. When either of the synthesized haptens was added to the tubes containing arsanilic-acid-egg white and its antiserum, the amount of specific precipitate formed was reduced, and when both synthesized haptens were added, the precipitate was completely suppressed. This showed that arsanilic acid was coupled to histidine in gelatin and both tyrosine and histidine in the egg white. This agrees with the known respective chemical structures, since gelatin contains histidine but not tyrosine, while egg white contains both of these amino acids. In this connection it should be remembered that the formation of a visible precipitate is not essential to the union of antigen and antibody, in fact it is a secondary reaction. It is evident that Landsteiner's suppression phenomenon is due to some form of interference on the part of the unattached hapten added to the antigen-antibody mixture. Apparently it is due to the fact that the formation of immune precipitates is diminished or prevented when the antigen is present in excess (Landsteiner, 1936, p. 119). Its value in determining the nature of an unknown hapten is well established.

Haptens Responsible for Cross-Reactions.—While some haptens are species-specific, many are not. The latter is indicated by the presence of the "A" and "B" factors of human cells, in the red cells of the anthropoid apes; of a hapten in one strain of Friedländer's bacillus sufficiently similar to the polysaccharide of Type II pneumococcus, as to be responsible for cross serological reactions; of a hapten in *Proteus* X19 so similar to one in the *Rickettsia* of typhus fever, as to make the Felix-Weil reaction possible. Such results enable one to interpret correctly numerous cross-reactions.

Pneumococcus Type-Specific Polysaccharide Serum Globulin Antigen.—Many immunological facts have been discovered as a result of extensive investigations of haptens carried on for many years. It has been found that when a pneumococcus type-specific polysaccharide is combined with suitable serum globulin there results an antigen which when injected into mice stimulates antibodies that protect the mouse against the pneumococcus corresponding to the hapten employed.

Spatial Relationships and Specificity Stereoisomers as Haptens.—The possibility that spatial relationship may be one way of

determining specificity is illustrated when one employs antigens having a common protein fraction and differing only in that their haptens are stereoisomers of each other. Thus when dextro-, levo-, and meso-tartaric acids are attached respectively to pig serum, three different antigens are formed. An antibody can be produced for each of these antigens that will not react with either of the other two.

Landsteiner's Method of Producing New Conjugated Antigens.—In order that the student may appreciate the significant work of Landsteiner on conjugated antigens, a brief discussion is given of the method he employs in introducing chemical groups (haptens) into an antigen. The discussion calls attention to the importance of these aromatic amino acids that have an OH group attached to the benzene ring in preparing a conjugated antigen. By first diazotizing the compound, the hapten can be linked to the nucleus replacing a hydrogen adjacent to the OH group. It is suggested also that linkage of the hapten with the salt-forming groups may occur. Specific antibodies representing each hapten-protein antigen can be produced.

Suggested Explanation of Drug Allergy.—Since it has been shown that an animal's own protein when modified by the addition of a hapten will stimulate antibodies if injected into the same animal, it has been suggested that some of the drug allergies occur when the drug, or some portion of it, acts like a hapten and unites with the patient's proteins and forms an antigen capable of stimulating antibody formation and sensitization of the patient.

Mosaic Structure of Antigens.—As one contemplates the possible significance of all these facts about antigens, one can appreciate perhaps the significance of Landsteiner's suggestion that antigens may have a mosaic structure. It is recommended that the student read Landsteiner's monograph on "The Specificity of Serological Reactions" (1936).

CHAPTER XXI

THE IMPORTANCE OF ANTIBODIES IN DIAGNOSIS

In the previous chapters antibodies have been defined and discussed and methods of measuring them have been suggested. While there is some controversy over their importance in the body's defense against infectious agents, there is general agreement that they are frequently of great value in diagnosis.

Discovery of Rôle of Agglutinins in Diagnosis.—THE WIDAL TEST.—Gruber and Durham (1896) investigated the phenomenon of bacterial clumping by immune serum and named it *agglutination*. Almost simultaneously Widal (1896) studied the blood of typhoid fever patients and found that such bloods specifically clumped suspensions of living or dead typhoid bacteria. Both Widal and Gruber recommended that the reaction be used as an aid in the diagnosis of typhoid fever. In honor of its discoverer, the test has been known as the Widal or the Gruber-Widal reaction.

SCOPE OF SUBSEQUENT INVESTIGATIONS.—Since then the phenomenon of agglutination has been investigated to ascertain the prevalence of normal agglutinins, the effect of vaccination on agglutinin titer, the time of their appearance and the titers attained in both experimental infections and clinical cases due to a variety of infectious agents. The agglutinin reaction has also found wide application in the identification of bacteria isolated from pathological lesions and other sources. When the test was first introduced it was not realized that serological types exist among the pneumococci, meningococci, streptococci and other bacteria. Perhaps this was because *B. typhosus* (*Eberthella typhosa*) was until recently regarded as being represented by one uniform type. While types are not as yet described for this organism, Downs (1925) and White (1926) have reviewed the literature and shown antigenic variation of practical importance. In preceding chapters a more comprehensive discussion of antigenic factors and variants now known to exist among bacteria have been discussed.

ANTIGENS USED IN THE WIDAL.—At this point it is sufficient to say that Widal discovered that suspensions of living organisms

(twenty-four-hour broth cultures or saline suspensions washed from a twenty-four-hour agar slant) or dead suspensions preserved with formalin were quite satisfactory for use in the test.

The suspensions should be diluted to match a satisfactory turbidity standard and tested for agglutinability by serums of known potency. The importance of flagellar and somatic antigenic factors is discussed later in this chapter. It is quite important that a smooth motile strain be used in antigen preparation.

TWO TECHNIQUES EMPLOYED.—There are two methods employed to determine whether the patient's serum will agglutinate at a di-

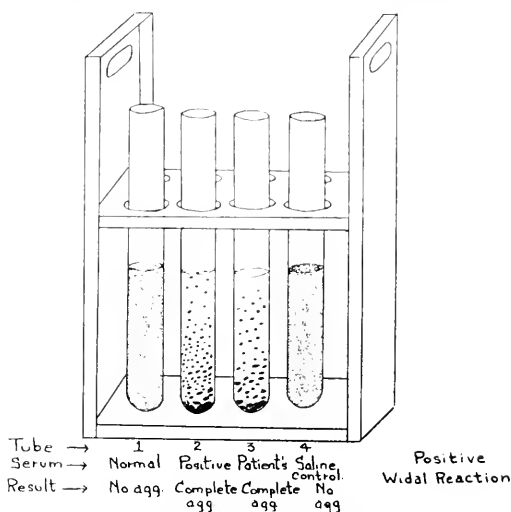


Fig. 15.—Positive Widal test.

agnostic titer suspension of *E. typhosa*. One is the macroscopic while the other is the microscopic technique. The former is usually the method of choice and the diagnostic titer used is as a rule 1-80. In carrying out the macroscopic Widal, one adds a uniform and measured amount of bacterial suspension to each of four vials or small test tubes. One then adds equivalent amounts of 1:40 dilution of normal human serum to the first, of 1:40 dilution of known positive human serum to the second, 1:40 dilution of patient's serum to the third, and saline to the fourth tube. This is indicated in Figs. 15 and 16. Where the agglutinability of the

suspension has been determined by sera of known potency, the positive human serum control is omitted.

INCUBATION, OBSERVATION AND INTERPRETATION OF RESULTS.—The tubes are shaken and incubated at 50°C . to 55°C . for two hours and then observed. These conditions favor the demonstration of H agglutinins. To demonstrate O agglutinins the incubation should be continued for 24 hours. The first tube containing normal serum mixed with bacteria should show uniform turbidity, while in the second containing known positive serum and suspension, one should see a complete or partial agglutination and settling out of

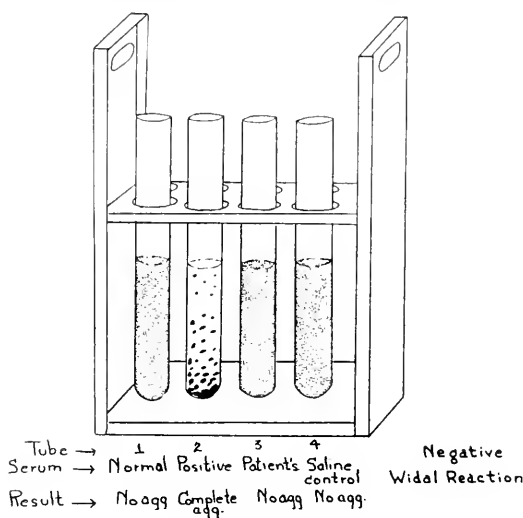


Fig. 16.—Negative Widal test.

clumps. The patient's serum is in tube three and this tube is to be compared with tubes one and two. If it is negative, it will be like the former, while if it is positive it will be like the latter. The saline control, tube four, should show uniform turbidity. Figs. 15 and 16 show positive and negative Widals, respectively.

MICROSCOPIC METHOD.—The microscopic method has inherent in it several sources of error to which exceptions are taken, but it does give some information not discoverable by the macroscopic test. In carrying out the microscopic technique, one prepares reagents as for the macroscopic test and studies them in hanging drops under the microscope. If living cultures are used, it will be ob-

served that in preparations containing normal serum or saline, the organisms remain motile and no clumps appear, while in preparations containing agglutinins the organisms lose their motility and come together in definite clumps.

It is also customary to test the patient's serum, in the same way, to see whether it agglutinates suspensions of *S. paratyphi* A or B* since these organisms produce clinical pictures that cannot be differentiated by physical examination and history of onset, although the course of each is usually milder and the duration shorter than in typhoid fever.

CONTROLS are very necessary in carrying out the test, since it is not impossible for the laboratory technician to get his cultures contaminated and use a suspension of the contaminant for the typhoid or paratyphoid suspension. Where the suspension is made from a pure culture of *E. typhosa* or of the *S. paratyphi* A and B,* the tubes containing known positive serum will show definite agglutination. The normal serum and saline controls should be negative to rule out spontaneous agglutination. When even pure cultures are used, if they are made from rough colony types, especially of *S. paratyphi* B,* they quite often show spontaneous agglutination. When this occurs, the normal serum and saline control tubes should show perceptible agglutination.

Aside from errors due to antigenic variation and technical procedure, there are others due to *mistakes in the interpretation* of the results. The Widal is essentially a test to ascertain whether specific agglutinins for *E. typhosa* or one of the paratyphoid organisms is present in the blood stream in sufficient amounts to give positive agglutination at the diagnostic titer used. If infection is present, the efficiency of the body tissues in the production of antibodies will determine the time of appearance and amount in the blood stream.

VARIATION IN TIME OF APPEARANCE OF AGGLUTININS.—Marked variation is to be observed in different individuals; some respond readily with early and abundant antibody production while others are poor antibody producers, the antibodies appearing later or in meager amounts. This accounts for the fact that the Widal may be positive in some cases of typhoid as early as the fifth day, while in others not until the third or fourth week. Gay (1918) feels

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that in approximately 100 per cent of the cases of typhoid fever, it will be positive at some time during the course of the disease.

Agglutinins in Carriers.—It should also be borne in mind that chronic carriers or persons who have been recently vaccinated against typhoid may have a sufficient amount of agglutinins in their blood to give a positive Widal. If they should develop influenza or a streptococcus endocarditis or miliary tuberculosis, or any other infection that gives a clinical picture similar to typhoid fever, the Widal might be positive and therefore lead to a mistaken diagnosis. Thus it will be seen that there are many possibilities of error not only in performing the test but also in the interpretation of the results.

To understand the significance of diagnostic tests based upon the presence of specific agglutinins in the blood of patients, one needs to know something of normal agglutinins and of the effect of vaccination and of infection on agglutinin titer.

Normal Agglutinins and Diagnostic Titers.—The blood of normal individuals not infrequently contains a small amount of agglutinins for various organisms. The normal agglutination titer for *E. typhosa* varied from 0 to 1:20 with an occasional finding somewhat higher, but with the average being about 1:5. Titers of 1:80 are considered of diagnostic value. In the case of *P. tularensis* or *Br. abortus* the normal agglutinating titer may be somewhat higher than for *E. typhosa*. Titers should be about 1:80 to suggest active infection with either of the organisms. In active infections one commonly finds a titer of at least 1:320 or higher. The occurrence of previous unrecognized infection is commonly mentioned as the explanation of the presence of normal agglutinins, but there are a few individuals who believe that in some cases these agglutinins may be normal physiological products just as the iso- and hetero-hemagglutinins are. Perhaps both explanations are valid.

Flagellar, Somatic and Labile Agglutinins.—In 1903, Smith and Reagh in their studies on a motile organism concluded that agglutinins for the flagella as well as for the bacterial cell (soma) were produced. This indicated that motile organisms possess flagellar antigens as well as somatic antigens. Felix and Weil (1917) although disagreeing with Smith and Reagh found two antigens which they named "H" and "O" respectively. The

former letter is now used to designate flagellar antigens, while the latter is applied to the somatic ones.

The extensive studies of Andrewes (1922, 1925) and of White (1926) have thrown a great deal of light upon the complex antigenic pattern of the *Salmonella* group. According to White, *E. typhosa* is related to *S. enteritidis* through a common somatic or O antigen.

Apparently the H antigens of *E. typhosa*, *S. paratyphi* A, and *S. enteritidis* are type specific, while the H antigen of *S. paratyphi* B, *S. aertrycke* and a few others are diphasic and may occur in one of two forms. They may change from one H form to another H form. The somatic antigens remain unaltered. The latter, however, as in the case of *S. enteritidis*, *E. typhosa*, *S. pullorum* and *S. sanguinarium*, may be shared by a number of organisms.

This somatic antigenic relationship between *E. typhosa* and *S. enteritidis* must be kept in mind in interpreting a Widal designed to test for fine-flocculating agglutinins.

The H antigen is contained in cultures showing motility. To prepare O antigens, advantage is taken of the solubility of the H antigens in alcohol. Bien and Gardner suggest adding an equal volume of alcohol to thick suspensions of the motile organisms and incubating the suspension at 37° C. for 24 to 36 hours. The alcohol destroys the H antigen. They are not injured, however, by formaldehyde.

In Chapter VI a description of "Vi" antigens discovered by Felix and Pitt is given. This is a labile surface antigen that is present in varying amounts in living virulent typhoid bacteria and tends to interfere with agglutination. It is thought to be responsible for inagglutinable strains of virulent *E. typhosa*.

Variations in Agglutination Titer Following Vaccination.—Information relative to the variation of agglutinin titer following vaccination has been obtained by studying the antibody response in the lower animals and in man following vaccination. Jörgensen and Madsen (1902), Levin (1902), Stäubli (1903) and others very early in this century made notable contributions along these lines.

More recently Felix (1924), Stuart and Krikorian (1928-29), and Dulaney, Wikle, Stewart, Rayfield, et al. (1933), have investigated the relative agglutinin response following vaccination

with typhoid vaccines. The last named authors find both agglutinins produced contrary to the conclusions of Felix who held that only "H" agglutinins were formed as a result of vaccination, while "O" agglutinins were produced by infection. In a series of individuals who were immunized against typhoid, the "H" agglutinin titer was much higher than the "O," the former showing a pronounced steady drop from an average titer of 1:95 for 20 weeks after the last injection followed by a more gradual decrease in titer until a titer of about 1:80 was reached in 140 weeks. The "O" agglutinations reached, on an average, a titer of only 1:80 and fell during the same period of time to approximately 1:40.

Effect of Allergy on Agglutinin Titer.—Jamson and Kessel (1932) noted low titers in a group of allergic individuals given multiple injections of typhoid vaccine. They assumed that immunity was produced in spite of the low antibody response. Inquiry should always be made of patients who are suspected of having typhoid fever to ascertain whether they have been vaccinated with a typhoid vaccine, since an increase in titer would be expected in such individuals.

Experimental Results on Agglutinin Variation.—The result of vaccinating a large series of rabbits, some being given one injection while others are given two, three or more injections, is quite surprising in that a wide variation in response is found. Some animals are very poor antibody producers, while others yield exceptionally high titers. If only one injection is given to an animal which produces antibodies *readily* the titer *may* change from zero on the day of vaccination to 1:500 or 1:700 on the fifth to seventh day, although often it is less. The concentration of antibody rarely continues to rise longer than the tenth day and frequently is dropping by that time to reach a low level in from three to six weeks. Where several injections of a vaccine are given, it is customary to administer them at from three- to seven-day intervals. This is done to keep the antibody titer rising. As a rule there is a slight drop in titer after each injection, followed by a fairly sharp rise during the next few days.

TIME OF APPEARANCE OF MAXIMUM TITER.—The maximum titer is usually attained after four to six injections and is reached usually five to seven days after the last injection.

With typhoid fever in an unvaccinated individual the agglutinin titer does not increase perceptibly until the fifth or seventh day of the disease. Occasionally it is the third week before an increase can be noted. The titer occasionally may not go above 1:100, but as a rule it will go much higher. Hence early in the disease when the bacteria are in the blood stream, a blood culture is the laboratory procedure of choice since the Widal is likely to be negative. During later weeks the opposite will hold true.

THE USE OF THE WIDAL IN VACCINATED INDIVIDUALS.—Where an examination is being made of the blood of an individual who has previously been vaccinated against *E. typhosa* the question arises as to whether the abnormally high titer is due to the vaccine or to an actual existing infection. It is not impossible for a vaccinated person to acquire an infection from massive doses such as may be present in contaminated milk. When such a question arises, it is answered by titrating the patient's blood daily using serial dilutions of his serum, and noting whether there is an increase in the titer, as one would expect if infection with *E. typhosa* is present, or whether the titer remains constant as it might after it had reached a low level several months following vaccination. Since infections due to *S. paratyphi A* and *S. paratyphi B** may resemble typhoid fever, it is customary to determine the agglutinin titer for each of these wherever a routine Widal is done.

Importance of "Fine Granular" and "Loose Flocculation."—In interpreting a positive diagnostic agglutination reaction there are those who believe that a "fine granular" type of agglutination indicates a favorable outcome, while a large loose flocculation is rather unfavorable. The present state of our knowledge relative to the rôle of antibodies in resistance to infection has been discussed in earlier chapters. In agglutination work care must be exercised to avoid overlooking fine granular agglutination.

TIME AND TEMPERATURE OF INCUBATION.—In regard to the time and temperature of incubation, it should be remembered that while incubation for several hours at room temperature or at 37° C. is frequently satisfactory for the Widal, 50° to 55° C. is preferable. The former conditions often give false negative reactions when one is dealing with suspensions of *Br. abortus* or with *P. tularensis* and for that matter with suspensions of the meningococcus used in

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carrier detection studies. In the study of the agglutination reaction with suspensions of these organisms, as well as with the Widal, it is recommended that an incubation of three to twelve hours at 55° C. as suggested by Weil (1905) be employed, and that readings be made again after the tubes have been standing in the refrigerator overnight.

Undulant Fever.—In undulant fever one has to bear in mind that there are at least three strains of *Brucella*: the caprine strain, the porcine strain and the bovine strain. While they differ in virulence for man, the caprine and porcine being quite virulent and the bovine strain less virulent, they are fortunately very similar antigenically. There are four laboratory procedures employed as aids in the diagnosis of Brucellosis. One of these is similar to the tuberculin test except that nucleoprotein obtained from *Brucella* organisms is substituted for tuberculin. A second test is based upon the phagocytic activity of the patient's leucocytes; this is called the opsonocytophagic test; a third test is based upon the isolation of *Brucella* organisms from the patient; and the fourth diagnostic laboratory procedure is a test for specific agglutinins. There is also frequently observed in undulant fever, an increase in agglutinins for *P. tularensis* just as the converse has been noted in tularemia; this will be discussed later in the chapter. Fairly high diagnostic titers are not exceptional. High normal titers might be expected if the ingestion of milk containing *Br. abortus* can occasionally produce unrecognized or mild infections as is claimed by many.

Heathman (1933) and others have shown that individuals employed in certain divisions of the meat industry show evidence of latent or unrecognized infection as evidenced either by positive skin reactions when tested with extracts of *Br. abortus* or by the presence of excessive amounts of agglutinin in their blood. She found that those working in the beef departments showed a high percentage of skin reactors, while agglutinin titers were high among those employed in the pork departments. Carpenter and Boak (1930) have also discussed the significance of antibodies in the diagnosis of undulant fever.

In the agglutination test employed in suspected *Brucella* infection of man and the lower animals two apparently equally reliable methods are in use. One is the test tube method similar to the

macroscopic Widal and the second is Huddleson's rapid spot plate method. Descriptions of these respective techniques are given by Kolmer and Boerner (1941) p. 596 or Gradwohl (1938) p. 923.

Tularemia.—Tularemia is a disease primarily of rodents and secondarily of man. Some valuable immunological studies can be made with this organism. It offers an interesting opportunity to study the agglutinin titer in uninfected tame rabbits which have never been exposed to infection as well as the titer in the normal,

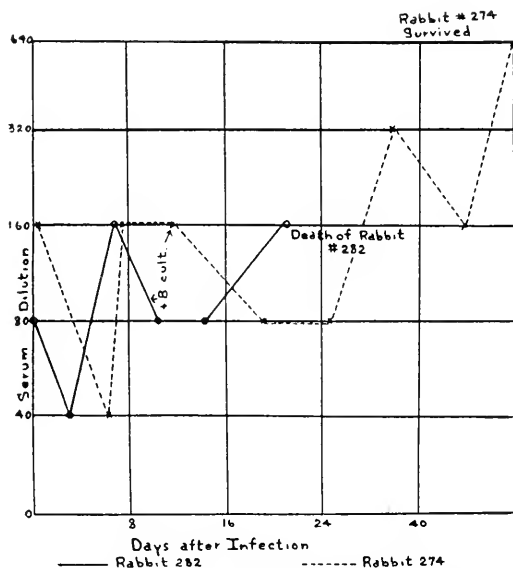


Fig. 17.—Graph showing fluctuation in agglutinins in partially immune and immune animals during infection with *P. tularensis*. (After Downs.)

partially immune and immune rabbits following and during infection. Downs (1932) has extensively investigated these phenomena.

AGGLUTININ RESPONSE TO INFECTION IN PARTIALLY IMMUNE ANIMALS.—Fig. 17 shows the fluctuations in agglutinin titer in a partially immune rabbit, Number 282, and a highly immune rabbit, Number 274, following the intradermal injection of a dose of living organisms that would kill a normal rabbit within a few days. It is interesting to note the drop of agglutinin titer simultaneously with the appearance of the bacteria in the blood stream.

It will be observed from a study of the graph that at the time both animals were inoculated with virulent organisms the partially immune rabbit's blood showed an agglutinin titer of 1:80 while blood from the immune rabbit (No. 274) had a titer of 1:160. The agglutinin titer of neither animal was constant during the succeeding days, but varied as indicated by the graph. Blood cultures were positive for the partially immune rabbit on the ninth day and for the immune animal on the twelfth day. In both animals there was a drop in titer associated with blood stream invasion and this was followed by an increase in titer.

It is interesting to note that the partially immune animal died on the eighteenth day when the titer had reached 1:160, which was twice as much as at the beginning of the infection, while the titer for the immune animal stayed low (1:80) between the sixteenth and the twenty-fourth day, when it began to increase. This lasted several days, then dropped and finally went up to 1:640 on the fortieth day after the initial infection.

INFECTION IN NORMAL ANIMALS.—The picture is quite different in the normal animal. It shows that blood stream invasion occurs much earlier and that no agglutinins can be demonstrated either initially or during the infection. The failure to demonstrate agglutinins is probably due to the fact that death usually occurs on the fifth or sixth day which is too short a time for agglutinins to appear. It is also interesting to note that the immune animal did not show a high titer (1:160) when infection was produced, but nevertheless, it survived blood stream invasion and completely recovered. These results show lack of accurate correlation between antibody titer and immunity.

The Effect of Specific Infection on the Titer of Agglutinin for Other Organisms.—There are many specific infectious diseases in which there occurs an increase in agglutinins for bacteria other than the causal agent. This phenomenon is of importance in typhus fever, tularemia, and also undulant fever.

FELIX-WEIL PHENOMENON.—Typhus fever is caused by certain exceedingly minute organisms called Rickettsiae. They have been successfully cultured by Nigg and Landsteiner (1932). Within the blood stream of typhus patients there appear agglutinins for a strain of *B. proteus vulgaris* called Proteus X19. The phenom-

enon of their appearance was first observed by Felix and Weil and is regarded as being sufficiently constant to be of value in the diagnosis of typhus fever. The agglutination of Protens X19 by the serum of a typhus fever patient is called the Felix-Weil reaction. Castaneda and Zia (1933) have demonstrated a common antigenic factor in Typhus Rickettsia and Protens X19. This accounts, apparently, for the presence of agglutinins for the latter.

TULAREMIA—SPECIFIC AND CROSS REACTIONS WITH *BR. ABORTUS* ANTIGENS.—In clinical cases of tularemia, it is not uncommon to observe an increase in agglutinins for *Br. abortus*. Conversely, in undulant fever, there may be an increase of agglutinins for *P. tularensis*. Francis (1926) has investigated the relative occurrence of agglutinins for *Br. abortus* in the blood stream of patients suffering from tularemia and has tabulated his results as shown in Table IX.

TABLE IX

U. S. PUBLIC HEALTH REPORTS, JUNE, 1926

CASE	TIME AFTER ONSET	TULARENSE	ABORTUS	MELITENSIS	TREATMENT OF SERUM
R. R. S.	18 days	640	40	40	Unheated glycerin
	26 days	1,280	1,280	640	Unheated glycerin
	7 months	640	320	320	55° C., no preservative
	9 months	640	320	320	55° C., no preservative
	1 year	320	320	320	55° C., trikresol
	1 year, 4 mo.	640	320	320	55° C., trikresol
B. F. T.	3 days	0	0	0	Unheated glycerin
	9 days	80	0	0	Unheated glycerin
	16 days	1,280	160	320	Unheated glycerin
	23 days	320	160	320	Unheated paracresol
	42 days	320	160	160	Unheated trikresol
E. W. M.	5 days	0	0	0	Unheated glycerin
	11 days	160	0	0	Unheated glycerin
	18 days	320	160	160	Unheated glycerin
	25 days	1,280	320	160	Unheated paracresol
	71 days	320	80	160	Unheated trikresol
	87 days	320	80	80	Unheated glycerin
J. W. G.	40 days	640	160	160	Unheated glycerin
	53 days	640	160	160	Unheated trikresol
A. M.	11 days	160	0		Unheated, no preservative
	24 days	2,560	160		Unheated, no preservative
	33 days	1,280	80	80	55° C., no preservative
	79 days	640	80	40	55° C., no preservative

Permission of author and Surgeon General, U. S. Public Health Service.

From an inspection of this table, it will be observed that patient R. R. S. developed agglutinin titers for *Br. abortus* quite comparable with those for *P. tularensis* and that the titer for *Br. melitensis* was noticeably increased. In the remaining four cases of tularemia, Francis records a definite increase in titer for both strains of *Brucella* organisms, but in no case did he observe such a striking response as in that of patient R. R. S. These results may be due to similar antigenic substances common to the *Brucella* group and to *P. tularensis* and also to the possibility that when tularemia develops in a patient who has a latent infection with some member of the *Brucella* group agglutinins for both result. Since the diagnosis of either of these diseases is usually confirmed by the agglutination test, an appreciation of such phenomena is of importance in interpreting laboratory reports.

ANTIGENIC RELATIONSHIPS BETWEEN *S. GALLINARUM* AND *E. TYPHOSA*.—The commercial laboratory is not infrequently called upon to test the blood of chickens for the presence of agglutinins for *S. gallinarum* or *S. pullorum*. Bushnell (1926) has recommended an agglutination procedure designed to determine whether active infection exists in the fowl. These organisms are of interest since they possess remarkable antigenic similarity to *E. typhosa*. Sherwood and Hoffman (1929) observed that the blood sera of several typhoid patients agglutinated a suspension of *S. gallinarum* (sanguinarum) at a higher titer (1:800) than the suspension of *E. typhosa* used in the Widal. Mulsow (1919) working in Jordan's laboratory concluded that the organisms could be differentiated only by careful absorption experiments. Theobald Smith (1915) has suggested that *E. typhosa* may be a variant of *S. gallinarum*. If this should eventually prove to be true, it might explain many things at present difficult to understand in regard to the epidemiology of typhoid fever. As mentioned earlier in this chapter, White (1926, 1932) reports the existence of a somatic antigen ("O" antigen) common to *E. typhosa*, *S. gallinarum*, *S. pullorum* and *S. enteritidis*. Attention has already been called to the discovery of carbohydrate-lipid complexes in *E. typhosa* by Topley, Raistrick and their associates (1933, 1938). These substances are highly antigenic and incite the production of the "granular" agglutinins.

The Agglutination Reaction in Other Specific Infectious and Toxemic Diseases.—**S. DYSENTERIAE.**—There are several strains of *S. dysenteriae* which differ antigenically. Infection with the Shiga strain induces agglutinins in the patient for that strain but not for the one described by Flexner. The converse also holds. Shiga discovered the dysentery bacillus by isolating various organisms from the stools of clinical cases and ascertaining which were agglutinated by the patient's serum.

GLANDERS.—In glanders, specific agglutinins develop as a rule for *P. mallei*. The agglutination test has found definite application in the diagnosis of this disease. Human infection with *P. mallei* is relatively rare. Whitmore (1913) described a glanders-like disease in man due to a new organism that has since been called *B. whitmori* by the English bacteriologists but named *Flavobacterium pseudomallei* by Bergey. Stanton and Fletcher (1925), Thompson (1933) and also Sherwood and Lan (1933) have observed antigenic relationships between *B. whitmori* and certain strains of *P. mallei*. The last named authors also noted some slight antigenic relationship between *B. whitmori* and an organism isolated by Sherwood from a case of meningitis in the central part of the United States. This latter organism has been named *Flavobacterium orchitidis* by Bergey. It is culturally and otherwise quite similar to *B. whitmori* except for the weak antigenic relationship. This is of interest since no strain of the latter organism has been isolated from any source outside of Asia.

ASIATIC CHOLERA, AND A FEW OTHER DISEASES.—The agglutination reaction is of little or no value in the diagnosis of Asiatic cholera, cholera carriers, diphtheria and scarlet fever because of the great irregularity in the appearance of agglutinins in these diseases.

Test for Heterophile Antibodies in Acute Infectious Mononucleosis.—The titer of normal heterohemagglutinins in human blood for sheep cells is usually less than 1:32. Paul and Bunnell (1932) discovered that this titer is increased quite extensively in most cases of acute infectious mononucleosis. Their discovery is now considered of diagnostic importance.

Identification by Agglutination or Precipitation.—In the study of bacteria isolated from various sources such as typhoid fever,

bacillary dysentery, Asiatic cholera, and the study of gram-negative diplococci from suspected meningococcus carriers and other conditions, it is often necessary to ascertain whether the organism isolated and culturally identified is agglutinated by known immune serum. If it is agglutinated by high dilutions of the known antiserum, its identity is considered as established. Thus if we isolate a motile gram-negative bacillus that is culturally similar to *E. typhosa*, the next step will be to see whether a suspension of the organism isolated is agglutinated by antityphoid serum and at a titer at which the antiserum will agglutinate a suspension of *E. typhosa*. In addition to this it has usually been decreed that for final identification an immune serum must be prepared against the new organism and this immune serum must react with a suspension of *E. typhosa* by agglutination and absorption tests in exactly the same way that it does with its homologous organism. This is the "mirror reaction."

BACTERIAL TYPES.—Since cellular antigens and specificity are discussed extensively in earlier chapters, it is sufficient to state at this time that each species of bacteria is represented usually by a number of types which can be differentiated from each other by agglutination. There are four or more types of meningococci, fifty-five specific types of pneumococci and an heterogeneous group not yet subdivided, four or more types of dysentery bacilli that also differ in some of their cultural reactions. The colon group is antigenically so diverse that the agglutination test is of no value in identification, hence one depends entirely upon morphology, staining, and cultural reactions. In regard to the staphylococci, only morphology, cell grouping and pigment production are used as a rule in identification. In the more recent work on streptococci (Bliss, Gordon, Tunnicliff, Lancefield, and others) it has been reported that hemolytic streptococci can be subdivided into a number of groups and types by agglutination or precipitation. Virulent organisms from human sources fall into Lancefield's group A. The green producing streptococci are more heterogeneous, i.e., show greater antigenic diversity.

C. DIPHTHERIAE.—In identifying *C. diphtheriae*, agglutination is not only of little value, but it is unnecessary since toxin production and specific neutralization of toxin by diphtheria antitoxin are excellent criteria for the final identification of the organism. All

strains produce the same kind of toxin, although they may show marked variation in agglutination.

CL. TETANI.—*Cl. tetani* has been divided into at least six types by the agglutination test but these, too, all produce one kind of toxin that is neutralizable by tetanus antitoxin.

CL. BOTULINUM.—An interesting exception to the rule that a single toxin is produced by all strains of an organism is to be found in *Cl. botulinum*. The three types of this anaerobe produce antigenically different toxins although all three toxins produce botulism in appropriate test animals. Toxin from Type A is neutralized only by antitoxin for Type A and not by antitoxin produced against the toxin of Type B or C. This holds true for each toxin. Neutralization and protection tests, rather than agglutination, are the final criteria in identifying these organisms.

E. TYPHOSA (*B. typhosus*).—*E. typhosa* shows a greater uniformity of antigenic properties possessed by various strains than any other organism. For this reason, the agglutination test, as previously mentioned, is quite generally used in the final identification of an organism resembling *E. typhosa* culturally and otherwise. Its somatic antigenic relationship to other organisms has already been discussed. Attention is called elsewhere to the relative inagglutinability of strains containing "Vi" antigen.

From the foregoing remarks, it will be seen that in determinative bacteriology, frequently many factors must be considered before final classification can be accomplished.

PNEUMOCOCCUS TYPING.—The pneumococcus is usually identified as a capsulated, gram-positive, lance-shaped diplococcus that is bile-soluble and pathogenic for white mice. By means of the agglutination and quellung reactions, pneumococci have been divided into fifty-five specific types such as I, II and III, etc., and an heterogeneous group. (Cooper et al., 1929-1932; Walter, Guevin and others, 1941.)

Reasons for Typing.—Specific type determination is done usually for three reasons: first, because some clinicians desire to treat certain types of pneumococcus pneumonia with immune serum specific for the type, for there is considerable evidence that it is of some therapeutic value especially in Type I infections. The results in Types II and III infections have been less favorable.

The second reason is to enable the clinician to make a more accurate prognosis and to have available accurate hospital records for future reference in regard to the prevalence of the various types, etc. Studies such as those of Avery, Chickering, Cole and Dochez (1917) are ample evidence of the importance of such a procedure. The third reason is that all research projects such as those of Avery and Goebel (1931), Heidelberger (1926) and others require accurate typing of the pneumococci with which they are working.

TECHNIQUE.—The technical procedures used in specific type determination were first suggested by Blake (1917) and Avery (1917). They consisted of a rapid method using calcium carbonate bloodbroth and the mouse method for agglutination and precipitation.

The quelling reaction has to a large extent replaced the agglutination and precipitation techniques. It is used either for the direct examination of spinal fluid, pleural exudates, in fresh sputum, or in connection with the mouse and cultural methods. When used to examine the peritoneal inflammatory exudates of mice inoculated with positive sputum, the quelling reaction gives excellent results.

Neufeld's Quelling Reaction.—In 1902 Neufeld reported that when specific immune serum is mixed with pneumococci, in the fresh state, there results not only agglutination but also a swelling (quellung) and the development of a ground glass appearance of the peripheral zone of the organisms. He added methylene blue to the mixture to stain the organisms. In 1932 Armstrong and also Logan and Smeall, using a slightly different technique, reported excellent results in typing. Their technique was found unsatisfactory by Sabin who reports excellent results with the Neufeld procedure. The latter was demonstrated to Sabin by Goodner. While the method is simple, rapid, and apparently accurate, it is obvious that the observer must be a good microscopist. Sabin's description of the technique is as follows:

Sabin's Description of Neufeld's Method.—“Two small flecks of sputum are placed on a cover slip (about 22 by 50 mm.) with a platinum loop, the diameter of which preferably does not exceed 2 mm. When the sputum is very tenacious, as it frequently is, it

may be necessary to use another wire for getting the sputum off the loop. To each bit of sputum an equal quantity of the undiluted rabbit serum* (Type I serum to one, and Type II serum to the other) and a loopful of standard alkaline methylene blue was added. A special deep, large, hollow-ground glass slide, big enough to cover both drops, was used; this special slide is convenient but not essential. The edges of the slide are smeared with petrolatum, it is placed over the cover slip, and the preparation is inverted. The examination is made with the oil-immersion lens and an artificial blue light. Although the reaction occurs almost immediately, it is best to delay the examination for about two minutes to allow for proper diffusion of the serum.

“It is important to observe that ordinarily, in the hanging drop preparation, pneumococci in sputum show no capsules; occasionally a faint halo of light but without any definite outline may be seen about the organism. However, in the type-specific mixtures of sputum and serum one finds the pneumococci surrounded by peripheral zones of characteristic appearance and distinct outline. This peripheral zone consists of a refractile substance which does not take the stain and which may be described as having a ground-glass appearance; the organism within it is stained blue. The size of this zone of ‘quellung’ varies in different sputums with organisms of the same type; generally, Type II pneumococci presented the larger zone. It is important to stress, however, that it is not so much the size of the zone as its characteristic appearance which determines a positive reaction. It is also necessary to state that this reaction does not depend on agglutination of the pneumococci, although this occasionally occurs in the sputum, but on the appearance of the individual organisms; furthermore, this appearance is so characteristic that the finding of even a single diplococcus which shows it is sufficient to diagnose the type, as shown in the accompanying illustrations.” (Figs. 18 and 19.) A more extensive discussion of pneumococcus typing is given by Kolmer and Boerner (1941, p. 447).

TYPING OF HEMOLYTIC STREPTOCOCCI has already been discussed in Chapter XIX. It will be recalled that Lancefield, by means of immune sera, has been able to divide the hemolytic streptococci

*Polyvalent serum representing known types is used before the final typing is done with individual specific type serum.

into groups and types. This work has assumed great importance because of the consistency with which group "A" streptococci are associated with human infection.

Skin Tests for Antibodies.—Other tests for the presence of antibodies or antibody-like substances are in common use: The

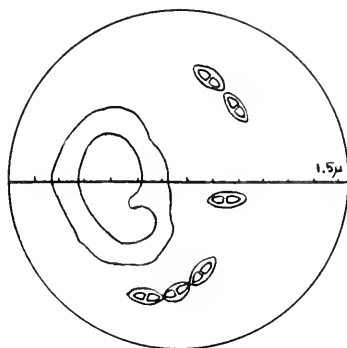


Fig. 18.—Type II pneumococcus in sputum mixed with Type I antiserum (rabbit); no "quellung." (After Sabin, J. A. M. A. 1933.)

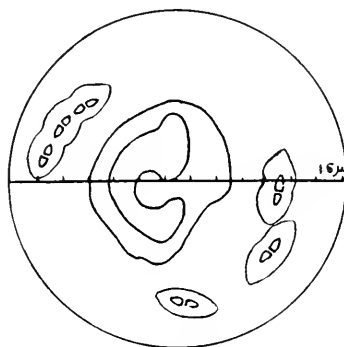


Fig. 19.—Type II pneumococcus in sputum mixed with Type II antiserum (rabbit); "quellung" reaction. (After Sabin, J. A. M. A. 1933.)

Schick test and Dick test for the presence of diphtheria and scarlet fever antitoxin respectively and the various tests for allergic reagins.

THE FRANCIS SKIN TEST.—This test, according to Edwards, Hoagland and Thompson (1939), consists of the injection of 0.05 c.c. of a dilution of 1:1,000 type specific pneumococcus polysaccharide in physiological saline intracutaneously on the flexor surface of the forearm. A positive result consists of an urticaria-like

wheel appearing at the site of injection within fifteen or twenty minutes. The appearance of pseudopods and extension of the circumference of the wheel are considered as definite evidence of a positive reaction. A positive test is an allergic reaction and indicates an excess of antibodies in the tissues. The test is used as a guide to the serum treatment of pneumonia since serum is discontinued in uncomplicated cases when the test becomes positive. Edwards et al. recommend that more serum be given if the reaction is doubtful. The authors state that the test as a guide in serum therapy rests upon a sound theoretical basis. They present an excellent discussion of the subject.

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CHAPTER XXII

THE BASIS OF BACTERIAL COMPLEMENT FIXATION TECHNIQUE

Introduction.—In the preceding chapters the subjects of antigens, antibodies and complement have been discussed along with our present concepts of specificity, cellular sensitization and the binding of complement by sensitized cells. It remained for Bordet and Gengou (1901) to discover that one could ascertain whether a given sample of serum contained antibodies for a specific bacterial antigen by mixing the two together with complement and later testing with sensitized red cells to see whether the complement had been bound. If the serum under investigation contained antibodies for the bacterial antigen, one would expect sensitization of the bacterial cells and a subsequent binding of complement. One could determine whether complement was bound or free by adding sensitized red cells, incubating and examining for hemolysis. Bordet and Gengou saw at once that such a procedure might be helpful in the diagnosis of certain specific infectious diseases to ascertain the kind of antibodies in the blood stream. They also realized that by this means an unknown organism might be identified as accurately as by the agglutination test. In the present chapter it is proposed to discuss the methods of performing the test and call attention to the reasons for adopting certain standard procedures.

The Original Bordet-Gengou Technique.—The original complement fixation technique was introduced by Bordet and Gengou in 1901 and can best be understood by a study of the accompanying protocol from their paper (Table X). These tubes were incubated for five hours at 15-20° C., and to each tube was added 0.2 c.c. of sensitized rabbit blood cells, and the tubes were shaken and incubated at 37° C., for one hour, when the results were read and recorded. The tubes appear as in Plate V.

It was found that in Tube A no hemolysis occurred after the sensitized red cells were added. The reason is presumably that the anti-pestis serum sensitized the bacteria in the pestis emulsion

TABLE X*

TUBE	COMPLEMENT	ANTIGEN	SERUM USED
A	0.2 c.c. guinea pig complement	0.4 c.c. pestis emulsion	1.2 c.c. anti-pestis serum (heated)
B	0.2 c.c. guinea pig complement	0.4 c.c. pestis emulsion	1.2 c.c. normal horse serum (heated)
C	0.2 c.c. guinea pig complement	None	1.2 c.c. anti-pestis serum (heated)
D	0.2 c.c. guinea pig complement	None	1.2 c.c. normal horse serum (heated)
E		0.4 c.c. pestis emulsion	1.2 c.c. anti-pestis serum (heated)
F		0.4 c.c. pestis emulsion	1.2 c.c. normal horse serum (heated)

*From Bordet and Gengou: *Ann. Inst. Pasteur* 15: 289, 1901.

and the sensitized cells combined with all of the complement, leaving none to act on the sensitized rabbit red cells when they were added. In Tube B complete hemolysis occurred since the normal serum could not sensitize the bacteria in the pestis emulsion and hence the complement remained free to act on the sensitized red cells when they were added. Tubes C and D are additional controls. They showed complete hemolysis since in the former there was no antigen (pestis emulsion) to be sensitized, while in the latter there was neither antigen nor immune serum, since normal serum had been substituted. No hemolysis occurred in Tubes E and F since neither contained complement. Similar results were obtained using an emulsion of typhoid bacilli and anti-typhoid immune serum in place of the pestis emulsion and its corresponding antiserum.

These authors also found that fresh human serum could be used as a substitute for guinea pig complement.

APPLICATION OF COMPLEMENT FIXATION.—It is obvious that this technique could be used either to identify an unknown organism or to ascertain whether antibodies for known organisms were present in the blood of patients. As an illustration of this latter possibility, Bordet and Gengou showed that an emulsion of *H. pertussis* when mixed with serum from whooping cough cases becomes sensitized, as shown by their capacity to fix complement.

At the present time bacterial complement fixation is used largely as an aid in the diagnosis of obscure gonococcal infections in the human being and for the detection of *P. mallei* infection in the

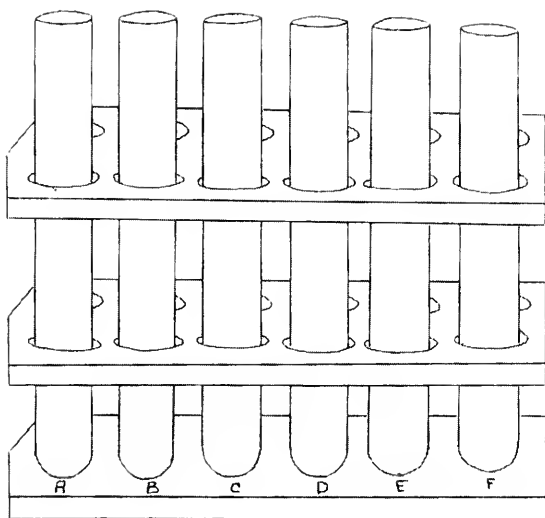


PLATE V.—ORIGINAL BORDET-GENGOU BACTERIAL COMPLEMENT FIXATION TEST

	A	B	C	D	E	F
Complement	0.2 c.c.	0.2 c.c.	0.2 c.c.	0.2 c.c.	-----	-----
Antigen	0.4 c.c.	0.4 c.c.	-----	-----	0.4 c.c.	0.4 c.c.
Antipestis serum (heated)	1.2 c.c.	-----	1.2 c.c.	-----	1.2 c.c.	-----
Normal horse serum (heated)	-----	1.2 c.c.	-----	1.2 c.c.	-----	1.2 c.c.

Incubate for fifteen to twenty hours at room temperature and add 0.2 c.c. sensitized red cells and incubate at 37° C. for one hour.

horse. It is used to some extent as an aid in the diagnosis of tuberculosis, but in general the tuberculin reaction has been found much more satisfactory. The agglutination reaction rather than complement fixation is used almost exclusively in the identification of unknown organisms and in the diagnosis of typhoid fever, undulant fever and tularemia. The complement fixation technique has found its greatest application as an aid in the diagnosis of syphilis. This will be discussed in Chapter XXIII. More recently Craig (1927, 1928, 1929, 1930) and Sherwood and Heathman (1932) have studied its use in amebic dysentery cases and carriers. Dulaney's* (1940, 1941) work suggests that the test may be of value in malaria; Lennette and Horsfall (1941) have employed complement fixation in their influenza work and Witebsky, Wels and Heide (1941) report that the complement fixation test is superior to the precipitin test in trichinosis.

REAGENTS AND FACTORS INVOLVED.—A study of the protocol illustrating the technique employed by Bordet and Gengou shows that the following seven reagents were used; namely—complement, bacterial antigen, bacterial antibody, normal serum, red blood cells, hemolytic amboceptor and physiological saline. It also involves certain arbitrary expressions of total volume in each tube, time, temperature and conditions of incubation as well as the use of test tubes and pipettes.

DEVELOPMENT OF MODERN TECHNIQUE.—Modern complement fixation is a result of intensive and extensive studies of each of these factors with a view toward obtaining greater accuracy, simplicity, and standardization of procedure. Its evolutionary development may be traced through the following progressive steps:

1. The introduction of a complement fixation technique by Bordet and Gengou in 1901. Their technique defined a unit of red cells (rabbit), specified physiological saline as a diluent, a primary incubation of 15 to 18 hours at 20° C., and a secondary incubation of one hour at 37° C. They apparently did not employ a uniform total volume nor did they recommend a method of titrating amboceptor, complement, or antigen.

2. Ehrlich, V. Dungern, Wassermann, Bruck and others introduced a standard total volume (Von Dungern preferred 2.0 c.c., Wassermann and Bruck 5.0 c.c.) and substituted sheep for rabbit

*Dulaney, A. D., and Stratman-Thomas, W. K.: Complement-Fixation in Malaria, *J. Immunol.* 39: 247, 257, 1940.

red cells, and introduced methods for titrating amboceptor and antigen. Wassermann and Bruck employed 1.0 c.c. of a 1:10 dilution of complement in their test. They also specified that both primary and secondary incubation be carried out at 37° C. The definition for the unit of amboceptor suggested by the Ehrlich school is essentially that employed today. While their *final* concentration of red cells was approximately the same as now employed, they did not prepare their suspension from packed cells.

3. Numerous modifications of the early technique have been used. Noguchi employed an antihuman hemolytic system and a total volume of 1.0 c.c. while the New York City and State Boards of Health have employed a total volume of 0.5 c.c. Others have employed various hemolytic systems and total volumes of 2.5, 3.0, 4.0, and 5.0 c.c. The Kolmer technique which is becoming more or less of a standard one in the United States specifies a total volume of 3.0 c.c.

4. The amount of complement used in the test has varied from 0.2 c.c. of undiluted complement employed by Bordet and Gengou, and a titrated amount of a 1:10 dilution employed by Noguchi to a titrated amount of a 1:30 employed by Kolmer.

5. While the time and temperatures employed in the *secondary* incubation have remained fairly constant since the beginning, the time interval and temperature of the primary incubation have varied. Bordet and Gengou used 15 to 24 hours at 20° C.; the Ehrlich school, Noguchi, Craig and others, one hour at 37° C.; and Kolmer, although not the first to suggest it, has employed 15 to 18 hours at 6°-8° C.

6. The amount of patient's serum employed in the test has varied from 1.2 c.c. suggested by Bordet and Gengou to 0.1 c.c. recommended by Noguchi and also by Kolmer until recently. Kolmer now employs 0.2 c.c. The time of inactivation of serum has varied from 15 minutes to 30 minutes at 56° C. The former time is recommended by Kolmer for the quantitative test and the latter time for the qualitative although he states that 30 minutes may be employed in preparing serum for either test.

7. Neither Bordet and Gengou nor the Ehrlich school attempted to standardize the glassware employed in the test as is done by

Kolmer. There are, however, good reasons for adopting standard equipment and standardized procedures. The pipettes he recommends enable the technician to combine accuracy with reasonable speed. The test tubes are of dimensions to insure a satisfactory height to the column of contents and permit adequate mixing of reagents by shaking the rack. There are equally good reasons for most of his other requirements.

8. In regard to the diluent used (0.85 per cent saline) in complement fixation work, it has remained essentially the same since its introduction by Bordet and Gengou with the exception that Kolmer adds 0.10 gm. of magnesium sulphate per liter. Since Kolmer has increased the sensitivity of his modification of the Bordet-Wassermann test by reducing the concentration of complement and by increasing the lipoid content of his antigens and by interpreting a one-plus reaction as positive, there has been a growing insistence upon the use of purer saline solutions.

9. As regards bacterial antigens to be employed in complement fixation considerable progress has been made but there is room for improvement. Bordet and Gengou used untreated bacterial suspensions while Wassermann and Bruck used bacterial extracts. Since then the New York City Board of Health has employed polyvalent antigens defatted with alcohol and ether; Wadsworth (1929) describes a "Dialyzed Distilled Water-Extract" method for the preparation of an antigen from tubercle bacilli; Price (1932) dissolves a suspension of the gonococcus in NaOH and later adds sufficient HCl to precipitate out an antigenic substance and Kolmer and Boerner (1941) describe other methods of bacterial antigen preparation. Torrey (1940) recommends Price's antigen for gonococcal complement fixation.

Theoretically every antigen should be tested to see if it is hemolytic, anticomplementary or antigenic, but Kolmer (1941) says that when the bacterial antigens are prepared according to his method they are rarely hemolytic and therefore the hemolytic titration may be safely omitted. For the sake of completeness we will include protocols of the hemolytic, anticomplementary and antigenic titrations as recommended by Kolmer. They are as follows:

HEMOLYTIC TITRATION OF BACTERIAL ANTIGEN

TUBE	ANTIGEN	HEATED HUMAN SERUM C.C. OF 1:10 DIL. OR SALINE	SA- LINE SOLN. C.C.	6° to 18° C. for 15 to 18 hr., followed by 30 min. in a water bath.	2% RED CELL SUSP. C.C.	Water bath for 1 hr.	HYPOTHETICAL READING
1	undil. susp.	0.5	1.5		0.5		25% hemolysis
2	1:2 dil.	0.5	1.5		0.5		trace of hemolysis
3	1:3 dil.	0.5	1.5		0.5		no hemolysis
4	1:4 dil.	0.5	1.5		0.5		no hemolysis
5	1:6 dil.	0.5	1.5		0.5		no hemolysis
6	1:6 dil.	0.5	1.5		0.5		no hemolysis

In the above protocol it will be noted that 0.5 c.c. of a 1:2 dilution of antigen was the least amount of antigen producing some hemolysis. Kolmer would call this the hemolytic unit.

The next protocol is given to illustrate one method Kolmer and others have used to determine the smallest amount of antigen that produces a slight inhibition of hemolysis. This amount is called the anticomplementary unit. The results are illustrated in Plate VI, Fig. 1, except that Tubes 11 and 12 are not shown on the plate.

The anticomplementary unit in the above protocol would be 0.5 c.c. of a 1:2 dilution. Kolmer regards the anticomplementary titration as very important and suggests that it be done at frequent intervals with most bacterial antigens.

The next protocol is an example of antigenic titration according to a method suggested by Kolmer. The results are illustrated in Plate VI, Fig. 2, except that the serum control Tube 11 and the control on the hemolytic system Tube 12 are omitted from Plate VI, Fig. 2.

In the accompanying protocol the antigenic unit, i.e., the least amount of antigen giving a ++++ fixation of complement (indicated by complete inhibition of hemolysis), is contained in Tube 8. This unit then is 0.5 c.c. of a 1:200 dilution of antigen. (See Plate VI, Fig. 2.)

If positive serum is not available, Kolmer suggests employing a dose of antigen in the test, equivalent to one-third the anticomplementary dose. Referring to the anticomplementary titer given earlier, it is found to be 0.5 c.c. of a 1:2 dilution of antigen.

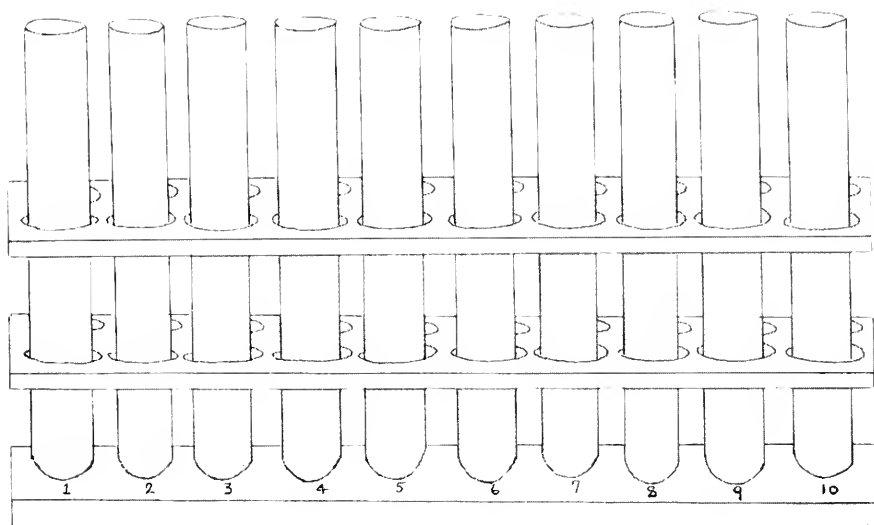


Fig. 1.—Anticomplementary titration of antigen in the presence of normal serum.

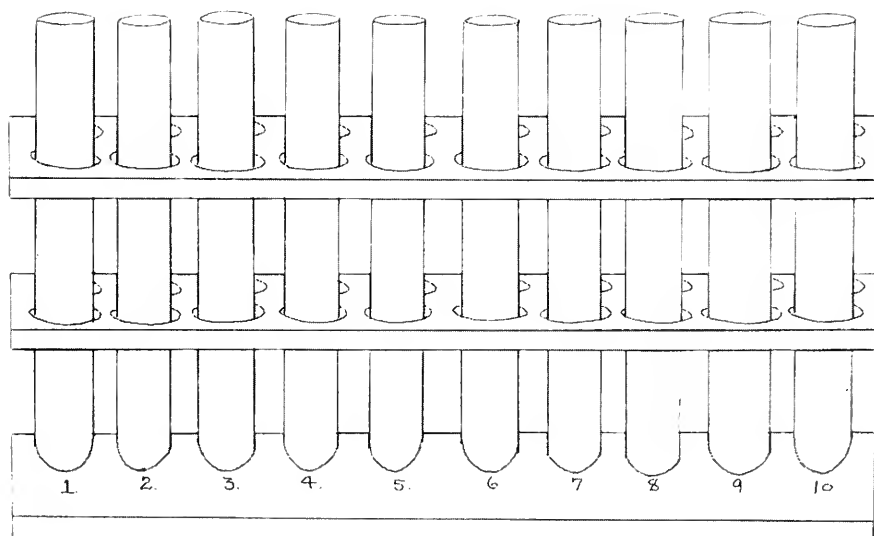


Fig. 2.—Antigenic titration in the presence of positive serum.

ANTICOMPLEMENTARY TITRATION

TUBE	ANTIGEN	HEATED NORMAL SERUM C.C. 1:10 DIL.	COMPLEMENT 2 FULL UNITS IN 1.0 C.C.	Incubate at 6° to 8° C. for 15 to 18 hr., followed by 30 min. in the water bath; or incubated in water bath only for 12 hr.	HEMOLYSIS 2 UNITS IN 0.5 C.C.	2 RED CELL SUSP. C.C.	EXAMPLE OF READING AS PER PLATE VII, FIG. 1
1	undil. susp.	0.5	1.0		0.5	0.5	no hemolysis
2	1:2 dil.	0.5	1.0		0.5	0.5	no hemolysis
3	1:3 dil.	0.5	1.0		0.5	0.5	complete hemolysis
4	1:4 dil.	0.5	1.0		0.5	0.5	complete hemolysis
5	1:6 dil.	0.5	1.0		0.5	0.5	complete hemolysis
6	1:8 dil.	0.5	1.0		0.5	0.5	complete hemolysis
7	1:10 dil.	0.5	1.0		0.5	0.5	complete hemolysis
8	1:12 dil.	0.5	1.0		0.5	0.5	complete hemolysis
9	1:16 dil.	0.5	1.0		0.5	0.5	complete hemolysis
10	1:20 dil.	0.5	1.0		0.5	0.5	complete hemolysis
11	None; 0.5 c.c. saline	0.5	1.0		0.5	0.5	complete hemolysis
12	None; 2.5 c.c. saline	None	None		None	0.5	no hemolysis

ANTIGENIC TITRATION

TUBE	DILUTION OF ANTIGEN SUSP. C.C.	HEATED POSITIVE SERUM C.C. OF 1:40 DIL.	COMPLEMENT 2 FULL UNITS IN 1.0 C.C.	Primary incubation at 6° to 8° C. for 15 to 18 hr., followed by water bath for 30 min.; or water bath only for 2 hr.	HEMOLYSIN 2 UNITS IN 0.5 C.C.	2% RED CELL SUSP.	Water bath for 1 hr.	READING OF TUBES
1	1:10	0.5	1.0		0.5	0.5		++
2	1:20	0.5	1.0		0.5	0.5		++
3	1:40	0.5	1.0		0.5	0.5		++
4	1:60	0.5	1.0		0.5	0.5		++
5	1:80	0.5	1.0		0.5	0.5		++
6	1:100	0.5	1.0		0.5	0.5		++
7	1:120	0.5	1.0		0.5	0.5		++
8	1:200	0.5	1.0		0.5	0.5		++
9	1:300	0.5	1.0		0.5	0.5		++
10	1:400	0.5	1.0		0.5	0.5		+
11	0.5 c.c. saline	0.5	1.0		0.5	0.5		-
12	1.0 c.c. saline	None	1.0		0.5	0.5		-

The test dose based upon using one-third of this would be 0.5 c.c. of a 1:6 dilution of antigen. If, instead of the above, antigenic titration was done and it was desired to use 10 antigenic units in the test, it would only be necessary to use 0.5 c.c. of a 1:20 dilution of this hypothetical antigen since one antigenic unit is 0.5 c.c. of 1:200 dilution.

After the antigenic titration has been completed and a test dose of antigen is decided upon, one is then ready to set up a complement fixation test to determine whether antibodies corresponding to the antigen are present in the patient's serum. In such a test it is necessary to establish by experiment (adequate controls) the validity of the results obtained. Three methods are available for complement fixation. One is the quantitative, a second is the qualitative, and third is the simplified test. A protocol for the simplified test is given in Table XI and is illustrated in Plate VII. In order for the student better to understand the significance of the results indicated in Table XI and Plate VII the following discussion is presented:

From a careful inspection of the above table it will be evident that Tube 5, which contains patient's serum, antigen, complement and sensitized red cells, constitutes the only test of the patient's serum for the presence of complement fixing antibodies. The other nine tubes are obviously controls. These controls are not only necessary from the standpoint of accuracy of results, but also frequently enable the laboratory worker to ascertain the cause of his failure.

NO HEMOLYSIS IN TUBE 5.—If either no hemolysis or partial hemolysis occurs in Tube 5 containing patient's serum, antigen, complement, red cells and hemolysin, the following questions arise:

1. Is it because the patient's serum contains sufficient antibodies to sensitize the antigen and thus give specific fixation?
2. Is the patient's serum anticomplementary?
3. Is the antigen anticomplementary?
4. Is it because of insufficient amount of complement due to a drop in titer or to errors in making up the standard dilution or to pipetting into the tubes?
5. Is it due to hemolysin deficiency for similar reasons?

TABLE XI
THE SIMPLIFIED BACTERIAL COMPLEMENT FIXATION TEST

TUBE	KNOWN + SERUM 1:2.5 DIL.	KNOWN NORMAL SERUM 1:2.5 DIL.	UNKNOWN PATIENT SERUM 1:2.5 DIL.	ANTIGEN	1:30 DIL. COMPLE- MENT C.C. 2 FULL UNITS IN 1.0 C.C.	1ST INCUBA- TION PERIOD FOR FIXATION OF COMPLE- MENT OVER- NIGHT IN ICE BOX	2% RED CELLS, 1 UNIT IN 0.5 C.C.	HEMOLYSIN 2 UNITS IN 0.5 C.C.	2ND IN- CUBA- TION	REMARKS
1	0.5 c.c.			5-10 units	1.0	6-18 hours' fixation at 6° C. to 8° C. followed by 10 to 15 min. at 37° C.	0.5 c.c.	0.5 c.c.	1 hour at 37° C. in water	Positive control for fixation
2	0.5 c.c.				1.0		0.5 c.c.	0.5 c.c.		Anticomplementary control for positive serum
3		0.5 c.c.		5-10 units	1.0		0.5 c.c.	0.5 c.c.		Anticomplementary control of normal serum and antigen
4		0.5 c.c.			1.0		0.5 c.c.	0.5 c.c.		Anticomplementary control for normal serum

5			0.5 c.c.	5-10 units	1.0		0.5 c.c.	0.5 c.c.	Experimental test for fixing property of patient's serum
6			0.5 c.c.		1.0		0.5 c.c.	0.5 c.c.	Anticomplementary control for patient's serum
7					1.0		0.5 c.c.	0.5 c.c.	Control of hemolytic system
8					None 2.0 c.c. saline		0.5 c.c.	0.5 c.c.	Control on hemolytic sensitizer
9				5-10 units			0.5 c.c.	0.5 c.c.	Antigen control for hemolytic properties
10					None 2.5 c.c. saline		0.5 c.c.	None	Salt solution control

6. Has the temperature of the incubator accidentally become so high as to destroy the complement during the period of primary incubation?

7. Were the tubes chemically clean and free from traces of acids, alkalies, dyes, etc., that might fix complement?

COMPLETE HEMOLYSIS IN TUBE 5.—On the other hand, if Tube 5, mentioned above, shows complete hemolysis, the following questions arise:

1. Is it because of a deficiency of antibody content?
2. Was the patient's serum inactivated?
3. Was too much complement added?
4. Will the antigen fix this particular amount and kind of complement in the presence of known antibodies?
5. Is the antigen hemolytic?
6. Are the red cells too fragile due to age?
7. Is the saline used hypotonic or isotonic?
8. Was it due to unusually slow fixation during the primary stage of incubation?
9. Is the hemolysis due to traces of acids, alkalies, or other reagents that might be present in the test tubes or pipettes?

OBJECT OF CONTROLS.—These nine control tubes are designed to answer most of the questions raised except those pertaining to the temperature of the incubator or to slow fixation during primary incubation. Acid hemolysis can be readily detected by the greenish discoloration of the liquid in the tubes.

RESULTS WHEN FIXATION IS DUE TO ANTIBODIES.—If fixation in Tube 5 is due only to the presence of antibodies in the patient's serum, then complete hemolysis should be present in serum control Tube 6, where antigen is omitted; complete fixation in Tube 1, the positive serum-antigen control; complete hemolysis in Tube 2, designed as a check on the anticomplementary nature of the positive serum; complete hemolysis in Tube 3 containing normal serum and antigen, and in its anticomplementary control Tube 4. Tube 7 should show complete hemolysis if the hemolytic system is working. Tubes 8, 9, and 10 should show no hemolysis if the hemolysis has been properly inactivated, if the antigen is not hemolytic, and if the saline is isotonic. In this protocol Tube 9 is used to determine if the antigen is hemolytic. Kolmer prefers to use it to ascertain if the antigen is anticomplementary and therefore includes 2 full units of complement. In that case complete hemolysis should

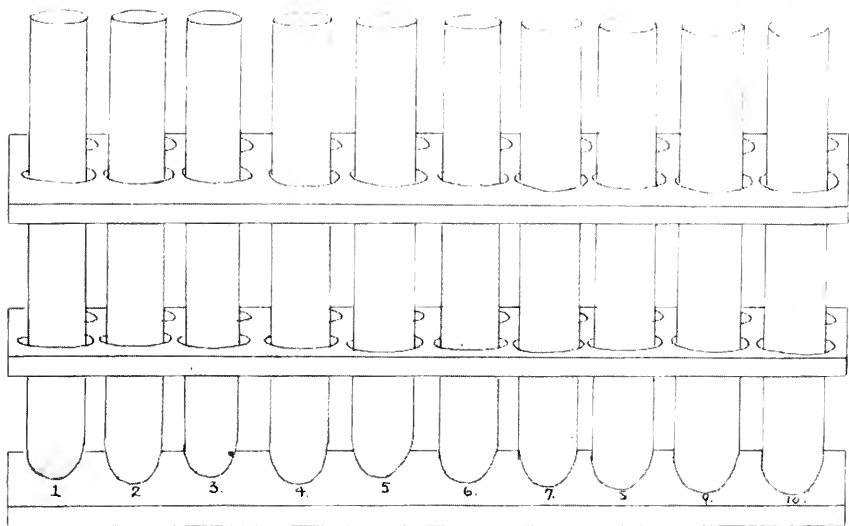


PLATE VII.—QUALITATIVE "ONE TUBE" BACTERIAL COMPLEMENT FIXATION COMPLETED TEST.

- Tube 1. Positive serum control (antibodies known to be present).
 2. Anticomplementary control on positive serum.
 3. Negative serum control (antibodies known to be absent).
 4. Anticomplementary control on negative serum.
 5. Patient's serum (the presence or absence of antibodies to be determined by this tube).
 6. Anticomplementary control on patient's serum.
 7. Control on hemolytic system.
 8. Control to determine if amboceptor is hemolytic.
 9. Antigen control (to determine if antigen is hemolytic).
 10. Control to determine if saline is hemolytic.

occur if the antigen is not anticomplementary. It is suggested that the student follow Kolmer's suggestions.

Quantitative and Qualitative Tests.—The technique of the quantitative test is similar to that of the simplified test except that in the former the serum is used in amounts of 0.2, 0.1, 0.05, 0.025, and 0.005 c.c. with 0.2 c.c. in the control tube. In the qualitative test Kolmer uses 0.2 and 0.1 c.c. and 0.2 c.c. in the control. Positive, negative and other controls should be included in both tests.

Reading the Results.—Kolmer states that the readings should be made immediately after the secondary incubation of one hour or they may be made 10 minutes after complete hemolysis occurs in the serum, antigen and hemolytic system controls.

Reporting Results.—Kolmer states that results may be reported as "positive," "doubtful" or "negative." He considers fixation of one plus or more as "positive"; the \pm reactions are called "doubtful" and when complete hemolysis occurs the report is "negative." Not infrequently the physician desires to know the degree of fixation observed. In such an event the report is given as four, three, two or one plus positive or \pm as doubtful and complete hemolysis as negative fixation.

Correlation With Clinical Findings.—In the case of gonococcal complement fixation, Price obtained 85 per cent positive results in known cases of infection. Park, Williams and Krumwiede report 75 per cent to 95 per cent positive results for complement fixation in clinically active pulmonary tuberculosis. In glandular tuberculosis and tuberculosis of the bones and joints, only 58 per cent and 22 per cent, respectively, of the sera gave positive results using complement fixation.

Discussion.—It should be remembered that the complement fixation test is just one of several techniques employed to detect antibody in blood serum and it is perhaps one of the most sensitive tests employed for that purpose. The reason for lack of correlation with clinical findings may be due to:

1. Defective reagents such as antigen, complement, hemolysin and saline.
2. Defective technique in preparing glassware or reagents, or errors in inactivation, incubation, or in setting up the test, etc.
3. The serum being tested.

From previous discussions bearing upon the complexity of cellular antigens and the effect of cultural environment, variation, heat and chemicals upon certain important substances associated with virulence it is obvious that obtaining a perfect antigen for complement fixation is not as yet possible. It is desirable to learn as much as possible about antigens so that better methods of preparations may be devised.

The difficulties that arise from defective technique are to a great extent within man's power to prevent. In this category are placed errors due to utter carelessness or ignorance of available knowledge concerning these tests. It is quite possible that as our understanding of the underlying physical and chemical mechanisms becomes more perfect our technique will improve.

The difficulties that are due to the serum under investigation are to a large extent beyond one's power of control. If the patient's tissue cells do not respond to antigenic stimulation with the production of antibody, then the negative results of complement fixation may be poor from the clinician's point of view but actually the results would be accurate from the standpoint of what the test is designed to accomplish, i.e., to test for specific antibodies. On the other hand the test might be positive in the case of a high *normal* antibody titer or when the antibody titer is increased due to vaccination, e.g., B. C. G. vaccine, and the positive results would not correlate with the true clinical condition of the patient. A few specific examples taken from actual laboratory records should cause the physician to realize that the lack of correlation between laboratory and clinical findings is not always due to faulty technique but that biological variation may be a factor.

VARIATION IN TITER OF HEMOLYSIN.—In the study of hemolysin production, 23 rabbits of about the same weight were used. Each received a daily intravenous injection of 0.4 c.c. of a 50 per cent sheep cell suspension over a period of 10 days. They were bled and the serum titrated for antibodies on the seventh to the ninth day after the last injection.

The antibody titers observed are interesting. Seven gave a titer of 1:10,000, two of 1:5,000, five of 1:2,500, three of 1:2,000, three of 1:1,500, one of 1:1,000, and two yielded a zero titer.

VARIATION IN AGGLUTININ AND PRECIPITIN TITER.—Similar results were obtained with two additional series used for precipitins

and bacterial agglutinins, respectively. It is realized that some of the variation may be due to variations in the time interval for maximum antibody production, but it is certain that some animals are entirely refractory as judged by our present methods of investigation.

In the commercial production of diphtheria antitoxin, horses are given repeated injections of toxin for the most part in the form of toxin-antitoxin mixtures or of anatoxin, which is toxin detoxified with formaldehyde. It has been the general experience that some horses yield little or no antitoxin while others produce high titered antitoxic sera with all degrees of variation between these two extremes.

ANTIBODY VARIATION DURING INFECTION.—Likewise, the physiological production of antibodies resulting from infection is variable even when the infectious agent is a good antigen. This is quite definitely illustrated by a study of the agglutinin response in normal rabbits infected with *P. tularensis* producing a disease common to rabbits. In a series of 11 normal rabbits, showing zero agglutinins before inoculation, and all surviving for almost the same length of time, Downs observed that one developed a titer of 1:1,200, three of 1:320, one of 1:160, two of 1:40, two of 1:20 and two continued with a zero titer until death. In the case of typhoid fever, the literature indicates that the Widal reaction is positive at some time during the disease in 80 to 92 per cent of the cases. Gay reported 100 per cent positive by the fifth week in a series that he studied. It should be remembered that *E. typhosa* is an unusually good antigen represented by practically one type in contrast to the numerous groups or types of pneumococci, gonococci, and meningococci found associated with their respective infections.

Summary of Recommendations.—In this chapter some of the factors influencing the results of bacterial complement fixation and the underlying philosophy of hemolysin, complement and antigen preparation and titration have been presented by discussion and protocols. In conclusion it would seem advisable to summarize most of the specific recommendations that have been presented relative to a standard procedure for complement fixation.

It will be observed that these conform very closely to Kolmer's recommendations:

1. The glassware, which includes pipettes, test tubes, volumetric flasks and graduates, should be chemically clean since minute traces of acids, alkalies, dyes, and many other substances may interfere with the successful performance of the tests.
2. The size of the test tubes should follow preferably Kolmer's recommendations, i.e., 15 mm. by 85 mm.
3. Sterile, cold, properly prepared physiological saline containing 0.1 gm. of magnesium sulphate per liter should be used uniformly as a diluent.
4. The final volume in each tube is to be 3.0 c.c.
5. One unit of cell suspension is contained in 0.5 c.c. of a 2 per cent suspension of sheep cells prepared from washed packed cells.
6. A unit of hemolysin (antisheep) is contained in 0.5 c.c. of the highest dilution that brings about complete hemolysis of a unit of red cells in the presence of 0.3 c.c. of a 1:30 dilution of complement when incubated for one hour at 37° C.
7. An exact unit of complement is the least amount of a standard dilution 1:30 of complement that will completely hemolyze a unit of red cells in the presence of 2 units of hemolysin when incubated for one hour at 37° C. in a water bath. A full unit is 0.05 c.c. more than a unit. Complement should be titrated in the presence of a test dose of antigen contained in 0.5 c.c. of saline.
8. Pooled complement from 3 or more guinea pigs is preferable to complement from one pig.
9. Complement may be preserved either by adding 10 times the normal concentration of dry NaCl or by freezing. A modified Sonnenschein method as suggested by Boerner and Lukens (1940) has been successfully used in this laboratory. Lyophil or cryochem complement is preferred.
10. High titered inactivated antisheep hemolysin is preferably preserved by adding an equal amount of C.P. glycerin.
11. For antigen titration 2 full units of complement and two units of hemolysin are recommended.
12. It is recommended that six to fifteen hours at 6° to 8° C. be used as the time and temperature for primary incubation.
13. For the second incubation it is recommended that one hour in a 37° C. water bath be employed.

14. The method of antigen preparation depends to some extent upon the organism used. It is suggested that the Kolmer or New York City or New York State Board of Health methods be used. For purposes of instruction defatted antigens are recommended.
15. An antigenic unit is the least amount of antigen or 0.5 c.c. of the highest dilution of antigen that gives complete (++++) fixation of 2 full units of complement in the presence of 0.1 c.c. of known positive human serum.
16. A satisfactory antigen must not be hemolytic or anticomplementary when 10 antigen units are present in the tubes.
17. All immune, positive, negative, and patient's sera are to be inactivated at 56° C. in a water bath for fifteen or twenty minutes for the quantitative test and thirty minutes for the simplified complement fixation test.

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CHAPTER XXIII

COMPLEMENT FIXATION IN SYPHILIS

History of Syphilis.—It is quite generally agreed that syphilis was unknown in Europe until about 1493. This date coincides with the return of Columbus from the West Indies. It is thought that members of his crew carried the disease from the West Indies to Europe. According to Williams, Rice and Lacayo (1927), syphilis made its appearance in Naples in 1495. It was called the “Neopolitan Disease” by the French, the “French Disease” by the Italians, and “Bubas” by the Spanish. In 1530, Fracastoro wrote a Latin poem which concerned a shepherd named Syphilus who became infected. The present name of the disease is attributed to this poem.

Etiology, Incubation and Primary Lesion.—Syphilis is caused by *Treponema pallidum*, discovered by Schaudinn and Hoffmann in 1905. Following infection, there is an incubation period of two, four or more weeks, after which there develops a small red papular lesion that enlarges to some extent and breaks down to form a small ulcer. This is the primary lesion, often called the “hard” or “Hunterian” chancre. It has raised edges, a grayish colored base, is freely movable and feels like a cartilaginous button when held between the thumb and forefinger. In the male it usually occurs on the sides of the frenum, on the glans, sulcus or prepuce and occasionally within the anterior portion of the urethra. The most frequent location of the primary lesion in the female is on the labia minora, os uteri, less frequently on the labia majora and rarely on the walls of the vagina. Extragenital chancres may occur. They are usually less indurated and may show greater ulceration.

While syphilis is usually transmitted by coitus or other methods of contact, it may also be congenitally acquired (intrauterine infection from the mother through the placenta). Third-generation syphilis has been reported. An excellent, brief discussion of the subject is given by Mitchell (1933).

Laboratory Procedure Indicated During Primary Stage.—During the primary stage of the disease the only dependable

laboratory procedure of value in the diagnosis of syphilis is one whose purpose is the demonstration of *Treponema pallidum* in the primary lesion. Since the index of refraction of the organism is such that it cannot be detected readily in a drop of exudate by direct microscopic examination nor stained by ordinary bacterial stains or even Wright's stain, it is obvious that other methods must be employed. *Treponema pallidum* can be identified definitely in a drop of exudate by means of the dark-field as suggested by Landsteiner, by staining with Fontana's silver nitrate stain, Giemsa's stain, or by mixing with a drop of India ink and making a smear in which the spirochetes stand out in contrast to the ink. Olsen and Weller (1932) state that it can be stained by ordinary bacterial stains if a phosphomolybdic acid mordant is used. It is not at all impossible for the inexperienced observer to identify erroneously other spirochetes as *Treponema pallidum*. The student can better appreciate this observation from a perusal of Noguchi's (1928) excellent monograph on Spirochetes. His photomicrographs are worthy of study.

BLOOD TESTS OF LITTLE VALUE DURING PRIMARY STAGE.—Serological tests of the patient's blood are of little or no value during the primary stage of the disease. Levaditi, La Roche and Yamaguchi (1908) according to Chesney (1927) obtained positive Wassermann reactions in a few individuals after the eighth day from the beginning of the primary stage, but it is generally admitted that negative reactions are quite common during this stage of the disease. This is because the complement fixation and flocculation tests are designed to detect a heterophile-like antibody called syphilitic reagin that appears in the blood stream in less than half of the infected individuals during the latter part of the primary stage of the disease. It is rarely present during the first week or ten days after the appearance of the primary lesion.

According to Denison and McDonald (1933) syphilitic reagin was detected in 40 per cent of 110 patients during the primary stage of syphilis. The Kolmer complement fixation test was positive in 36.3 per cent, while the Kahn was positive in 41.8 per cent. The average results obtained by the use of four different antigens yielded the figure of 40 per cent given above. The authors do not

state the number of days that elapsed from the appearance of the primary lesion until the blood was taken for serological examination.

The value of the Kline (1931) flocculation test of exudates obtained from the primary lesion is yet to be determined.

Secondary Stage.—**TIME OF APPEARANCE OF SYMPTOMS, LABORATORY FINDINGS AND DURATION.**—The secondary stage of the disease makes its appearance about four to six weeks after the development of the primary lesion. It is spoken of as the stage of generalized infection and is characterized by fever, rash, sore throat, a more extensive involvement of the lymphatics, and a secondary anemia. Other secondary manifestations such as alopecia (loss of hair), mucous patches, etc., may occur. The spirochetes are widely disseminated in the body at the time the secondary lesions appear and are present not only in the blood but also in all of the secondary lesions. Syphilitic reagin is found in the blood in 85 to 90 per cent of individuals during the secondary stage of the disease where treatment has not been instituted.

COMPLEMENT FIXATION AND PRECIPITIN TESTS IN UNTREATED CASES AND TREATED CASES.—Denison and McDonald (1933) report positive serological findings in 85.2 per cent of 108 untreated and 46.3 per cent of 54 treated patients showing secondary manifestations. In this series the Kolmer complement fixation test was positive in 81.6 per cent and the Kahn in 85.4 per cent of the untreated cases. In the treated cases the former was positive in 40.7 per cent and the latter (Kahn) in 48.2 per cent. Roby (1933) believes that all untreated cases of secondary syphilis will be found positive by the Wassermann test.

It is stated frequently that the secondary stage lasts about two years. This, however, is subject to considerable variation. As the acute symptoms subside, the organisms disappear from the general circulation and become localized in one or more tissues. The lesions that subsequently develop give rise to the symptoms of tertiary syphilis.

Syphilis May Clinically Resemble Almost Any Disease.—It is generally appreciated by clinicians that syphilis may manifest itself clinically in such diverse ways as to resemble almost any other known disease. This is well expressed in the saying of Osler,

“Know syphilis in all its manifestations and relations and all other things clinical will be added unto you.” It is obvious that any brief description of syphilitic manifestations such as is given in this chapter is exceedingly inadequate and is mentioned only to enable the student to appreciate the function, value and significance of laboratory tests in the diagnosis of syphilis.

Syphilis Characterized by Periods of Latency Followed by Periods of Activity.—The disease is also especially characterized by periods of latency followed by development of symptoms. Thus the onset of the tertiary stage is given as from two to ten years or more after infection, although it has been reported as early as six months. Its duration is not limited to any number of months or years. Periods of quiescence and recurrence are common. The symptoms are due to chronic inflammation of various tissues. A very common tertiary lesion is the gumma which resembles the tubercle of tuberculosis. It is a granuloma due to infection and may occur in any tissue of the body. Tertiary lesions of the skin, mucous membranes, circulatory system (aneurysms, endarteritis), lungs, etc., may also be observed.

In the pregnant female, miscarriages commonly occur or there may be intrauterine infection of the fetus without miscarriage, but with resulting congenital manifestation.

Serology of Umbilical Bloods.—Rohy (1933) considers that the results of umbilical blood Wassermann reactions may be relied upon as an index of the presence or absence of syphilitic reagin in the mother's blood at the time of labor. If the umbilical cord Wassermann is positive, it indicates that the mother has and the child may have syphilis. If the latter has the disease, it will continue to show a positive blood Wassermann. On the other hand, if the reagin but not the spirochetes have been transmitted from the mother, the child does not have syphilis and the blood Wassermann will become negative within two months. He states that his results, obtained independently, are confirmatory of similar data reported by Dunham (1932).

Tertiary Syphilis.—**SEROLOGICAL FINDINGS.**—During the tertiary stage of the disease, negative serological tests for the presence of syphilitic reagin in the blood are quite frequent. Denison and McDonald report on 672 untreated and 1,408 treated cases of tertiary syphilis. Of the untreated cases, 37.9 per cent gave posi-

tive serological findings as compared with 45.8 per cent of the treated cases. The Kolmer complement fixation test was positive in 32.6 per cent of the untreated and 34.0 per cent of the treated cases, while the Kahn precipitation test was positive in 41.3 per cent of the former and 49.8 per cent of the latter.

In a series of 889 *treated* cases of syphilis in which the stage of the disease is not mentioned, Heathman and Higgenbotham (1932) found the Kolmer-Wassermann complement fixation test positive in 63.1 per cent, the Kahn in 66.7 per cent and the Kline in 73.8 per cent. In 150 *untreated* cases of syphilis, the Kolmer-Wassermann test was positive in 88.6 per cent, the Kahn and Kline flocculation tests in 90.6 and 92.0 per cent, respectively.

TABES, PARESIS, AND CEREBROSPINAL LUES LATE MANIFESTATIONS OF TERTIARY STAGE OF SYPHILIS.—Other late tertiary manifestations of syphilis, considered by some as symptomatic of a quaternary stage or parasyphilis, are due to involvement of the central nervous system. These are represented by tabes dorsalis, paresis and cerebrospinal lues. It is generally agreed that spinal fluid examinations are of paramount importance in obtaining laboratory evidence of syphilis of the nervous system. Blood tests may be negative, while spinal fluid examinations may give definite laboratory evidence of syphilis. Nonne, according to Levinson (1919), reported both blood and spinal fluid Wassermann tests positive in 100 per cent of his cases of general paresis. In tabes without paresis, the blood was positive in 60 to 70 per cent, in cerebrospinal lues it was positive in 80 to 90 per cent, while the spinal fluid was positive in 100 per cent of both in the series he reported. His data were obtained before Kolmer introduced his modification of the Wassermann test or Kahn and Kline had perfected their modifications of earlier flocculation tests.

The series of events leading up to the introduction of the complement fixation technique as an aid in the diagnosis of syphilis form an interesting chapter in the history of diagnostic serology.

Wassermann and Bruck Modification of the Bordet-Gengou Technique for Bacterial Complement Fixation.—In 1905 Wassermann and Bruck were interested in finding some method of standardizing meningococcus immune serum and finally reported that a modification of the Bordet-Gengou complement fixation technique gave satisfactory results. The modified procedure which

they recommended involved a number of striking departures from the original technique. These differences are set forth in the following short paragraphs since the technique was later to form the basis of the Wassermann reaction.

TOTAL VOLUME.—In the Bordet-Gengou technique the total volume was 2.0 c.c. in some tubes while in others it was 1.6 and 1.8 c.c., respectively. Wassermann and Bruck recommended that it be 5.0 c.c. in all tubes.

BACTERIAL ANTIGENS.—In the original technique the antigen consisted of a suspension of bacteria of which an arbitrary amount was used in the test. Wassermann and Bruck recommended that *extracts* of bacteria be used and that the antigenic unit be determined by titration and that two units be used in the test.

COMPLEMENT.—Where 0.2 c.c. of guinea pig or human serum was used in the original test, the modification called for 1.0 c.c. of a 1:10 dilution of guinea pig complement. Apparently an arbitrary rather than a titrated amount was used in both instances.

IMMUNE OR PATIENT'S SERUM.—Bordet and Gengou used 1.2 c.c. of inactivated immune serum or normal serum in each tube, while Wassermann and Bruck used 1.0 c.c. of a 1:10 dilution of inactivated serum. They found that large amounts of serum as recommended by Bordet and Gengou are anticomplementary.

RED CELL SUSPENSION.—In the Bordet-Gengou technique 0.2 c.c. of a 10 per cent suspension of sensitized rabbit cells was added to each tube. Since the total volume, while variable, was approximately 2.0 c.c., the final concentration of red cells was about 1.0 per cent. Wassermann and Bruck added 1.0 c.c. of a 5 per cent washed sheep red cell suspension to each tube, and since the total volume adopted by them was 5.0 c.c., the final concentration would likewise be 1.0 per cent.

HEMOLYSIN.—In the original technique the red cells were sensitized before they were added to the tubes, while in the Wassermann-Bruck modification the hemolysin was titrated and two units were added to the tubes containing 1.0 c.c. of 5 per cent sheep cell suspension.

The Application of Complement Fixation to the Diagnosis of Syphilis.—During the same year (1905) that Wassermann and

Bruck suggested this modification of the original Bordet-Gengou technique for use in bacterial complement fixation, Schaudinn and Hoffmann discovered *Treponema pallidum*, the cause of syphilis. It occurred to Wassermann, Neisser and Bruck that complement fixation might be used in the diagnosis of syphilis if a suspension of *Treponema pallidum* could be obtained for use as an antigen. Since the organism could not be cultivated at the time, it was finally decided to make a saline extract of syphilitic tissues rich in spirochetes. The first work was done on the blood of apes in which syphilis had been produced experimentally. Wassermann, Neisser and Bruck prepared what they regarded as a specific *Treponema pallidum* antigen by making a saline extract of a syphilitic fetal liver rich in spirochetes. The technique employed was essentially the Wassermann-Bruck modification of the Bordet-Gengou bacterial complement fixation technique except for the method of antigen preparation and the controls which this modification made necessary.

CONTROLS EMPLOYED IN THE ORIGINAL WASSERMANN TECHNIQUE.

—The original Wassermann technique for the diagnosis of syphilis included many controls, a number of which are no longer employed. Citron (1909) has summarized the controls used in the original technique somewhat as follows:

1. The first control was to ascertain whether an aqueous extract of normal tissue (liver) and patient's serum would bind complement. It was believed that the fixation of complement depended upon spirochetes being present in the antigen and corresponding antibodies in the serum. Hence, this control tube should show no fixation of complement, as spirochetes were not present in the normal liver.

2. The second control was to show that known positive syphilitic serum when mixed with an aqueous extract of a syphilitic fetal liver containing spirochetes would fix complement.

3. The third control was to show that positive syphilitic serum would not fix complement in the presence of an aqueous extract of normal tissue (liver).

- 4 and 5. The fourth and fifth controls were to show that normal serum when mixed with an aqueous extract of either a syphilitic or normal liver respectively would not fix complement.

6 and 7. These controls were to demonstrate that aqueous extracts of syphilitic and normal liver respectively are not anti-complementary.

8, 9 and 10. These controls were to ascertain whether the patient's serum, known positive and negative sera were anticomplementary.

In addition to these there was included a control on the hemolytic system and also on alcoholic extracts of both syphilitic and normal tissues when these were employed.

Wassermann's first paper appeared in 1906 and contained results that were encouraging but far from satisfactory. Wassermann, Neisser, Bruck and Schucht (1906) and Wassermann and Plant (1906) made additional reports upon the new method of diagnosis of syphilis. They concluded that it was a specific complement fixation test for spirochetal antibodies in the patient's serum and that it possessed diagnostic value.

IMPORTANCE OF CITRON'S WORK.—Citron (1907, 1908, 1909, 1912) was apparently one of the first to confirm their results and to make extensive use of the test as an aid in the diagnosis of syphilis in man.

FIRST STAGE OF THE DEVELOPMENT OF THE WASSERMANN REACTION.—Kolmer (1922) speaks of three stages in the development of the Wassermann reaction. The first was the one just discussed in which the reaction was considered as due to the fixation of complement by spirochetes that had been sensitized by specific antibodies present in the patient's blood serum.

SECOND STAGE BEGAN WITH THE USE OF ALCOHOLIC EXTRACTS OF GUINEA PIG HEART.—The second stage began in 1906, 1907, when Landsteiner and Stanković (1906), Marie and Levaditi (1907) and Landsteiner, Müller and Pötzl (1907) showed that alcoholic extracts of normal guinea pig heart as well as syphilitic tissues could be used successfully as an antigen in the complement fixation test for syphilis. This led to extensive chemical studies of these alcoholic extracts and to a realization that lipoids and not spirochetes were sensitized by an antibody-like substance in the serum of syphilitic patients and that this lipid-antibody complex was responsible for the fixation of complement in the Wassermann test. H. Sachs and others attempted to introduce artificial antigens with indifferent results except for the acetone-insoluble lipid

antigen suggested by Noguchi and Bronfenbrenner (1911). Many modifications such as those of Tschernogubow (1908), Noguchi (1909), Hecht (1909), Stern (1909) and Bauer (1909) were introduced. At the present time these are for the most part of historic interest only and the student is referred to the original articles for further information.

THIRD STAGE INTRODUCED BY CHOLESTERINIZED ANTIGEN.—The third stage, according to Kolmer (1922), began with the discovery by Browning, Cruikshank and McKenzie (1910) that cholesterol added to lecithin antigen improved it and also the discovery of H. Sachs that a cholesterolized alcoholic extract of guinea pig heart is a better antigen than a plain alcoholic extract. These observations were soon confirmed by McIntosh and Fildes (1912), Walker and Swift (1913), and Kolmer, Laubach and Williams (1914). In all Wassermann tests for many years it has been customary to use three antigens, a cholesterolized, a plain alcoholic extract of normal heart and an acetone-insoluble lipid antigen. False positives have been encountered occasionally when only cholesterolized antigens were employed, but fairly accurate results are obtained when all three antigens are used and the results carefully interpreted.

FOURTH STAGE: INTRODUCTION OF IMPROVED ANTIGEN.—While Kolmer speaks of only three stages in the development of the Wassermann reaction, it seems desirable to mention two or three additional stages of development.

A fourth stage representing definite improvement in the antigen occurred when Neymann and Gager (1917) made their recommendations as to antigen preparation. These recommendations included a change from guinea pig heart, which contains Forssmann's antigen, to beef heart, which does not contain the heterophile antigen. Since occasionally one encounters a normal serum with a high heterophile antibody titer, it is conceivable that false positives may, on rare occasions, be obtained. They further recommended the preparation of an alcoholic extract of beef heart using methods of grinding and drying, preliminary and final extractions of the tissue and later cholesterolizing part of the extract, that are quite similar to the more recent methods recommended by Kolmer. They further suggested that a cholesterolized and an acetone-insoluble lipid antigen be prepared from portions

of the plain alcoholic extract of beef heart, thus insuring a certain uniformity in lipid content of the three antigens.

FIFTH STAGE: STANDARDIZATION OF WASSERMANN REACTION BY KOLMER.—The fifth stage began with the extensive investigations of every aspect of the complement fixation test for syphilis by Kolmer and his colleagues in 1919 and ended with his recommendations for a standard qualitative and quantitative Wassermann technique in 1922 and 1925. These are generally spoken of as the Kolmer or Kolmer-Wassermann qualitative or quantitative complement fixation reactions in syphilis.

A sixth stage exists at the present time and is one of revaluation, reconciliation, reinvestigation, and coordination. The complement fixation test for syphilis is assuming greater value in all laboratories where an unmodified Kolmer technique is employed. Clinical pathologists who have either published or adopted various modifications of the Wassermann technique have been slow to abandon these methods and adopt Kolmer's recommendations in their entirety, but a perusal of the literature seems to indicate that there is a slow but definite trend in that direction. The American Public Health Association is actively interested in the standardization of the Wassermann reaction as evidenced by the fact that it has a committee of which Ruth Gilbert is chairman, working toward this end.

An extensive literature that has been augmented recently shows renewed interest in the physicochemical aspects of complement fixation and may lead to further improvement in technique. There is also a growing conviction among serologists that a flocculation test such as the Kahn or Kline should be done simultaneously with the Kolmer complement fixation test on each serum.

In order that the student may appreciate the value of the extensive investigations of the Wassermann reaction carried out by Kolmer, a list of his publications leading to the first Kolmer complement fixation test is included in references at the end of the chapter.

Kolmer Test.—In the preceding chapter (XXII) the technique is given for Kolmer's bacterial complement fixation test. Except for the differences in antigens the technique for the Kolmer modification of the Wassermann used in the diagnosis of syphilis is the same. It is described in detail in United States Public

Health Supplement No. 11 to Venereal Disease information. This can be obtained from the Superintendent of Documents at a nominal cost. Since the Public Health service plans to issue revisions of Supplement No. 11 from time to time it would seem advisable for medical students, and others interested, to be on the alert for such revisions.

The *antigen* used in the Kolmer test is a cholesterolized and lecithinized alcoholic extract of heart muscle. It is prepared by extracting powdered beef-heart with acetone for 5 days to remove all the acetone soluble substances since they are thought to be undesirable. The acetone extract is filtered off and discarded. The dry residue is next extracted with chemically pure absolute ethyl alcohol for 5 days and filtered. This filtrate is saved since it contains the acetone insoluble, alcohol soluble lipoids used in the Kolmer antigen. To improve the antigen, i.e., to make it more sensitive, Kolmer adds 0.2 gm. of cholesterol to each 100 c.c. of the alcoholic solution of lipoids. After shaking and heating for one hour in a water bath at 55° C. to aid in the solution of the cholesterol it is allowed to stand at room temperature for 2 or 3 days and finally filtered to remove any precipitates that may have formed. This cholesterolized alcoholic extract is standard stock solution of antigen.

Kolmer also prepares a *new antigen* which is more sensitive than the above. The only difference is that the new antigen has not only all of the lipoids of the first antigen but there has been added to it, just before he added the cholesterol, all of the ether soluble but acetone insoluble lipoids obtained from the first 4 ether extracts of heart muscle that are usually discarded in the preparation of either Kahn or Eagle antigen. These added lipoids make the new Kolmer antigen more sensitive.

TITRATION OF ANTIGEN.—Both of the antigens mentioned are titrated in the same way. Kolmer says that it is unnecessary to titrate these antigens for hemolytic or anticomplementary units. It is, however, necessary to titrate for antigenic activity. This he does in accordance with the method of Boerner and Lukens. A brief summary and protocol is as follows:

He first prepares a 1:80 dilution of antigen by adding, drop by drop, 0.1 c.c. of the *cholesterinized* alcoholic solution of lipoids to 7.9 c.c. of saline, shaking between each drop. Beginning with this

he prepares the following higher dilutions, 1:160, 1:320, 1:640, 1:1,280 and 1:2,560.

He then prepares 5 dilutions of inactivated positive serum in such a way that by pipetting 0.5 c.c. of each he can arrange six sets of 5 test tubes in a rack with the amounts of serum shown in the following protocol. Each tube also has 0.5 c.c. of the antigen dilution indicated.

ANTIGEN TITRATION

SERUM IN 0.5 C.C.	ANTIGEN IN 0.5 C.C. AMOUNTS					
	1:80	1:160	1:320	1:640	1:1280	1:2560
0.005	-	-	++	-	-	-
0.0125	-	+	++++	+++	++	+
0.025	+	++++	++++	++++	++++	+
0.05	+++	++++	++++	++++	++++	++
0.10	++++	++++	+++	+++	++++	+++

Kolmer defines the dose of antigen to be used in the Kolmer complement fixation test as the largest amount of antigen giving a 4+ reaction with the smallest amount of serum. He says that, if three dilutions all give 4 plus reactions with the smallest amount of serum, one should choose a dose midway between the highest and lowest. In the above protocol the test dose would be 0.5 c.c. of a 1:320 dilution.

As mentioned previously in this chapter the technique of the Kolmer complement fixation test used to detect syphilitic reagin is the same as that described in the previous chapter for bacterial complement fixation except for the antigens used. The lipoid antigen is substituted for the bacterial antigen in the Kolmer modification of the Wassermann.

The tests are also reported the same. It will be recalled that the committee on the Evaluation of Serodiagnostic Tests for Syphilis of the United States Public Health Service cooperating with the American Society of Clinical Pathologists recommend that four plus, three plus, two plus or one plus reactions in the first tube be reported positive while plus or minus be called doubtful and complete hemolysis negative. Kolmer, however, prefers to call four plus and three reactions in the first tube as *strongly positive*, two plus reactions or one plus reactions as *weakly positive* and plus-minus as *doubtful* reactions and complete hemolysis in first tube as negative.

For a more complete description of the technique the student should read Supplement No. 11 issued in June, 1940.

Kilduffe's Ten Basic Principles in Serological Diagnosis.—In regard to the present status of the serological diagnosis, Kilduffe* (1933) discusses ten basic principles which he regards as important. His observations are worthy of serious consideration and are summarized as follows:

“1. The diagnosis of syphilis, however achieved, should be surrounded by every possible safeguard, regardless of the time, the labor, the expense of the minutiae involved.

“2. The diagnosis of syphilis should be based upon a careful, well-balanced consideration of all the data, however obtained, rather than predicated solely upon one or two isolated particularized facts or findings.

“3. A careful and intelligent study of syphilis is impossible without constant recourse to laboratory avenues of investigation, especially serological studies.

“4. Laboratory procedures must be regarded solely as constituting a single phase in the examination of the patient.

“5. A joint and interlocking responsibility rests upon both serologist and clinician entitling each to demand somewhat of the other and obligating both to a joint obligation of their combined resources in the interest of the patient.”

6. He states that in his opinion the Kolmer complement fixation test (quantitative) is one of the most valuable laboratory procedures available, “but it must be said that this applies only when the method is used as described without distortion of its principles or evasion or omission of its essential minutiae.

“7. So great is the practical specificity of precipitation tests under properly controlled conditions, that they may be accepted as valuable additions to the serological study of syphilis, and as useful, if not essential, adjuncts to the complement fixation test.

“8. While both the complement fixation and the precipitation reaction are biologically nonspecific, they possess, nevertheless, an extraordinary degree of practical specificity when properly performed under carefully controlled technical conditions, so much so, that positive reactions are consistently encountered in only one disease other than syphilis, namely yaws.

*Kilduffe: *Am. J. Clin. Path.* 3: 61, 1933.

"9. There is no serological procedure at present available, nor is it probable that one will ever be devised, with which a false negative reaction may not be obtained.

"10. The simultaneous use of both complement fixation and precipitation tests on every serum should be practiced routinely."

This paper by Kilduffe (1933) as well as the reports of Denison and McDonald (1933), Heathman and Higginbotham (1932) and Nigg and Larsen (1928), previously mentioned, as well as many others indicate that there is a growing appreciation of the value of the Kohler-Wassermann complement fixation technique and of the need for a standardized procedure that will undoubtedly include some form of flocculation test as a check on the former.

The Provocative Wassermann Reaction.—THE EFFECT OF TREATMENT ON THE REAGIN CONTENT OF THE BLOOD. An excellent discussion of the provocative Wassermann reaction is given in a paper by Belding (1929). He says that Gennerich (1910) and Milian (1910) reported, almost simultaneously, that in a certain percentage of syphilitics with negative blood Wassermanns, the complement fixation becomes positive following a single injection of arsphenamine. Others have reported various drugs as well as foreign protein as apparently producing provocative reactions. The average dose of arsphenamine recommended is apparently 0.3 Gm. Belding says that a few investigators believe that the phenomenon called a "provocative Wassermann reaction" is a myth. They explain the positive results as due either to errors in laboratory technique or to a coincidence in the occurrence of reagin following the injection of a drug or foreign protein. While Belding concludes that the majority of provocative reactions reported in the literature may be "ascribed to technical error" he reports that 3.3 per cent of 338 patients under treatment for syphilis showed an increase in reagin content of the blood following the first treatment. He regards the test as of doubtful value because of the many possibilities of error.

Wassermann-Fast Cases.—An excellent discussion of Wassermann-fast syphilis is given in a paper by Tobias (1928). He says that the term includes both those cases that are apparently refractory to treatment as indicated by the persistence of symptoms and a positive Wassermann, and second, cases that become, after adequate treatment, free of all symptoms except a positive blood Wasser-

mann. His paper is devoted to a discussion of the latter group. He reports that 48 or 13 per cent of 364 consecutive cases of syphilis of all types appeared to be Wassermann-fast. Of this series 11 per cent were of tertiary or late syphilis, 15 per cent latent, 30 per cent neurosyphilis, and 40 per cent congenital. He regards it as inadvisable to consider a case Wassermann-fast unless the complement fixation test is positive after four or more courses of treatment. In his opinion, these are all cases of incompletely cured syphilis. Whether such a conclusion is correct is a disputed point among clinicians.

Effect of Malarial and Diathermy Treatment.—Nicole and Fitzgerald (1931) report on a series of malarially treated cases of general paralysis. Before treatment was instituted, 100 per cent had a positive spinal fluid and 89 per cent a positive blood. After treatment there were 66.7 per cent whose spinal fluids and 70 per cent whose blood sera showed the presence of syphilitic reagin as determined by complement fixation.

Epstein and Paul (1933) report upon a series of cases of neurosyphilis treated by means of diathermy. They state that the serologic changes were not striking considering the group as a whole. An excellent short review of the use of diathermy in the treatment of syphilis is given by Schamberg and Butterworth (1932) and O'Leary and others (1940). They report some cases showing definite serological improvement, although clinical improvement occurred in others without demonstrable blood changes.

Immunity to Syphilis.—The subject of immunity in syphilis is well reviewed by Chesney (1927), Zinsser, Enders and Fothergill (1939) and others to whom they refer. The following brief summary of present concepts may be of interest to the student:

1. Syphilis seems to be a purely human disease that may be transmitted artificially to monkeys and rabbits. Zinsser (1939) says that the reported transmission of syphilis to the llama is not borne out by subsequent investigation.

2. There is no evidence of natural, individual or racial immunity.

3. In acquired syphilis, reinfection is possible during the incubation period and most of the primary stage of the disease. A refractory stage develops which, all agree, persists during the secondary stage and in many cases much longer.

4. Neisser maintains that the refractory stage or immunity to reinoculation is evidence of an existing infection, but Chesney (1927) concludes that a definite immunity persists after the body is free of infectious agents.

5. Clinical evidence indicates that reinfection is rarely observed. This question is also discussed by Cannon (1933) in a report of a case of reinfection.

6. A number of syphilologists hold that antibodies do not seem to play a rôle in acquired immunity to *Treponema pallidum*. Two mechanisms are suggested by Neisser. One of these is a condition of cellular indifference to the presence of the spirochetes. This phenomenon he has named "anergy." The second is a state of allergy or hypersensitivity that may function in the body's defense.

7. There is some controversy over the rôle of phagocytosis in immunity to *Treponema pallidum*. Chesney (1927) cites the investigations of both Ehrmann and Levaditi who report having observed phagocytosis of *Treponema pallidum* in stained sections. Both Zinsser (1939) and Chesney (1927) suggest that perhaps the reticulo-endothelial system may play a rôle in acquired immunity in syphilis.

Nonspecific Wassermann Reactions.—It is generally admitted that false positive Wassermann reactions may occur in such conditions as acute infectious mononucleosis, malaria, leprosy and perhaps a few other diseases. Landsteiner and Van der Scheer (1927) say that it is known that rabbits infected with trypanosomes develop positive Wassermann reactions. They report that similar positive results were obtained by injecting dead trypanosomes into rabbits.

Kemp, Fitzgerald and Shepard (1940) have summarized the literature bearing upon the occurrence of positive serological tests for syphilis in animals other than man. It is evident that positive serological tests, especially flocculation tests, are of frequent occurrence in many species of animals. Their blood contains a normal biologic reagin.

Whether genuine nonspecific reactions occur in man has been the object of a recent survey by the United States Public Health Service. Eagle (1941) summarizes the results of this study involving 40,545 initial specimens taken from as many college students representing twenty-five schools. All specimens giving posi-

tive reactions were re-checked. The incidence of positive tests in these students, all of whom were nonsyphilitic, as judged by history and physical examination, was approximately 1:1,125. As Eagle says, at first sight, this incidence appears disturbingly high. However, when the results are analyzed in the light of the incidence of known syphilis in the respective campuses he found a rather high correlation between the number of known cases of syphilis and the number of apparent false positive reactors.

When these facts are taken into consideration a revised estimate would suggest that the number of biologic false positives was not over 1 in 4,000 students. It should be remembered that these data have been obtained only for the age group of college students. Our results (Sherwood, Bond and Canuteson, 1941) on 1,018 students conformed quite closely to the results obtained for the whole group.

Kahn (1941) reports that the normal biologic reagin can be differentiated from syphilitic reagin by a new flocculation technique which he calls the *Verification test*. He finds that animal and human sera that give false positive reaction will show stronger reactions at 1° C. than at 37° C. whereas syphilitic sera give weaker or negative reactions at 1° C. and stronger reactions at 37° C. This test will be described briefly in the next chapter.

Mechanism of the Wassermann Reaction.—The mechanism of the Wassermann reaction is discussed extensively by Wells (1929), Zinsser (1931), Eagle (1929, 1930, 1931) and others. The exact origin and nature of syphilitic reagin is at present unknown. While it has the properties of an antibody, it has been generally regarded as not specific for *Treponema pallidum* as Wassermann originally thought, but consistently sensitizes certain lipoids when they are properly dispersed in saline. Eagle and Hogan (1940), however, regard the reagin as specific for a lipoid common to both animal tissue and *Treponema pallidum*. According to Wells (1929) Sachs suggests that perhaps infection with *Treponema pallidum* causes the liberation or formation in the body of lipo-proteins for which the tissues produce an antibody which we call reagin. He conceives of the lipoid or hapten portion as present in the tissues of many animals and that reagin will sensitize such a hapten antigen *in vitro* just as the haptens previously discussed will react *in vitro* with homologous immune serum.

The mechanism of complement fixation may be discussed with reference to plain alcoholic and cholesterolized antigens, respectively. When one adds a plain alcoholic extract of heart muscle to saline the lipoids are dispersed in the saline and to some extent peptized by it. Those finely dispersed lipid particles can adsorb reagin globulin and the resulting complex will bind complement.

When a cholesterolized alcoholic extract is added to saline, the cholesterol is dispersed as fine particles more rapidly than the lipoids present. The latter are, however, dispersed and adsorbed by the cholesterol particles. The lipoids form more or less of a surface film over the latter. Because of the presence of cholesterol, there is little or no peptization of lipoids by the saline. For this reason the sensitivity of the antigen is increased. The lipid-coated particles of cholesterol adsorb syphilitic reagin from positive serum and the resulting antigen-reagin complex adsorbs complement in the same way that antigen-antibody complexes are known to adsorb or fix complement.

Summary.—In this chapter a large amount of material has been presented in relatively few pages. It is obviously impossible to summarize condensed material of this type adequately. To facilitate a rapid review of the chapter the following conclusions and observations may be suggestive:

1. Syphilis was probably introduced into Europe from the West Indies about 1493. It acquired its name from a Latin poem written by Fracastoro in 1530.

2. The etiological factor is *Treponema pallidum* discovered by Schaudinn and Hoffmann in 1905.

3. In the primary stage of syphilis, only laboratory methods designed to detect the parasite are of value in diagnosis.

4. In the secondary and later stages of the disease, the laboratory methods used as an aid in the diagnosis of syphilis are those designed to detect syphilitic reagin in the blood or spinal fluid of the patient. The frequency of occurrence of syphilitic reagin in the various stages of syphilis is discussed. The data given are based upon complement fixation and flocculation tests.

5. In 1905, Wassermann and Bruck became interested in the bacterial complement fixation test previously discussed. They modified the Bordet-Gengou technique and undoubtedly improved its efficiency. As soon as it was established that *Treponema pal-*

lidum is the cause of syphilis, it occurred to Wassermann that this disease might be diagnosed by bacterial complement fixation. He and his colleagues prepared an antigen by extracting a syphilitic fetal liver, rich in spirochetes, with saline. They did this five years before Noguchi taught the scientific world how to cultivate *Treponema pallidum*. They found that the saline extract possessed some antigenic property as measured by complement fixation. In their opinion this complement-fixing property depended upon the spirochetes present. Within the next year, Marie and Levaditi (1907) and also Landsteiner, Müller and Pötzl (1907) showed that spirochetes are not the antigenic factors in the Wassermann antigen but that alcohol-soluble lipoids from normal heart muscle and other tissues are the substances that adsorb syphilitic reagin and thus bind complement.

6. Six stages in the development of our present serological methods used in the diagnosis of syphilis are next discussed. These include the original Wassermann technique and concepts; the discovery of lipid antigens; the discovery that cholesterol increases the sensitivity of lipid antigens; the modification of Neymann and Gager; the modifications of Kolmer and the present stage in which a general standardization of the Wassermann based upon extensive experimental, clinical and statistical studies is nearly completed. The flocculation test is becoming an accepted adjunct to the Wassermann test.

7. The various experimental studies of Kolmer and his colleagues are summarized and discussed. Likewise the recommendations of the Committee on Adherence to Conventional Technique in the Performance of Reliable Serologic Tests for Syphilis. Kilduffe's ten basic principles governing the serological diagnosis of syphilis are also presented.

8. The student is made acquainted with the importance of umbilical cord Wassermanns and of the meaning of the terms "Wassermann-fast" and "provocative Wassermann." The effect of diathermy and malarial treatment on the blood Wassermann is also mentioned.

9. Reference is made to Chesney's (1927) excellent monograph on *Immunity in Syphilis* and to a recent paper on reinfection in syphilis. Chesney seems to approve of Neisser's views that "anergy" and "allergy" are important factors in the body's

defense against reinfection. Both Chesney (1927) and Zinsser, Enders and Fothergill (1939) state that the reticulo-endothelial system may also be an important defensive mechanism.

10. A discussion of nonspecific Wassermann reactions is given.

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CHAPTER XXIV

PRECIPITIN TESTS IN SYPHILIS

Introduction.—Many precipitation tests applicable to syphilitic serum have been proposed since the observation by Michaelis (1907) of a precipitate when heated syphilitic serum and lipoidal tissue extracts were mixed. This method has been described by various authors as a precipitation, flocculation or a clarification reaction. In the serum of syphilitics there is usually present an antibody-like substance, reagin, which is directly or indirectly responsible for changes in the dispersion of the lipoidal antigens. These aggregates may vary in size from small microscopic to large macroscopic clumps. In normal human serum reagin is rarely present, hence the "reagin-lipoid" complex cannot be formed. Thus there is no increase in the size of the dispersed particles of lipoidal suspension. The precipitation tests, like complement fixation methods, are a qualitative or a quantitative estimation of "reagin" resulting from the infection and not a test for the presence of the causative organism.

Historical Development.—The successful development of the precipitation methods began with the work of Sachs and Georgi, and Meinicke, hence their practical application has been within the last twenty years. It is of historical interest to note that the first attempt to apply precipitation methods followed the Wassermann, Neisser and Bruck (1906) complement fixation method by one year. Michaelis (1907) using the Wassermann antigen originated this early test, believing he was using a specific extract of spirochetes and securing a specific precipitin test for the spirochete. This was followed by Klausner's (1908) method of precipitation with distilled water and the taurocholate suspensions. These early precipitation tests were macroscopic test tube reactions.

INTRODUCTION OF CHOLESTEROL.—Jacobstahl made a long step forward by introducing an alcoholic extraction of syphilitic liver as an antigen in place of the lipid-poor saline extracts. His test was also the first microscopic reaction. This test depended upon

the formation of an ultramicroscopic precipitate. While the complicated procedure involved in the Jacobstahl method was impractical, it stimulated extensive research in this field. Bruck and Hidaka (1911) modified this method so that the precipitate was microscopic rather than ultramicroscopic in character. Meinicke (1917, 1918, 1920) followed with several modifications. In the same period Sachs and Georgi (1920) suggested cholesterol as an added substance to increase the sensitivity of the tissue extracts. Although the Sachs-Georgi reaction has been generally discarded, their principle of adding cholesterol or a similar sensitizer to reinforce the tissue extract has made the practical development of the flocculation test possible. Many tests have been and are being proposed with the objective of increasing specificity or sensitivity or both, but thus far they are modifications of this original method.

Simplicity of Flocculation Method.—The simplicity of the flocculation methods contrasts sharply with many of the proposed complement fixation procedures. The methods combine high sensitivity and specificity, rapid results and stable standardized reagents at a low cost per test. It further permits the use of several methods as a check on each other with economy of time.

Status of Flocculation Tests.—The value of the precipitation method for the detection of syphilitic reagin is still a controversial subject. However, the widespread acceptance of such tests as the Kahn, Meinicke, Kline and others is an expression of approval by most syphilologists. From the present state of confusion generally standardized precipitation methods will develop which have adequate confirmation and of a specificity and sensitivity consistent with clinical practice. While many of the modifications are commendable, the process of standardizing has progressed slowly, due to the many technical modifications in use. It is difficult to pick an ideal test with such an assortment from which to choose when each offers one or more characteristic advantages.

Principle of Flocculation Tests.—The basic mechanism of all precipitation tests is essentially the same. They differ in points of technique, sensitivity, and interpretation. A positive reaction is characterized by the formation of aggregates which result from the combination of the lipoid suspension and "reagin" found in syphilitic serum but not present in nonsyphilitic serum. The

"antigen" used to detect syphilitic reagin consists of a dispersion of lipoidal substances in an aqueous suspending fluid (continuous phase). The reaction between the reagin in the serum and the particle of the "antigen" (the discontinuous phase) occurs at the surface interface of the particle. There is deposited a film of "antibody globulin" around the particle with a corresponding alteration in solubility of the interfacial film through an "antigen-antibody complex" formation with behavior like a denatured protein. This results in a reduction of the potential difference between the particles, when electrolytes are present in the aqueous phase. The mixture is agitated in some mechanical manner so that the particles come in contact with one another. These particles are held together, after contact is once made, by their cohesive force in the absence of a high potential difference. A high potential difference would cause the charged particles to repel each other.

In the negative reaction no clumping of particles occurs. There are no changes in the P.D. and the like charged particles repel one another with no resulting changes in dispersion.

The agglutinated particles may vary from a finely granular microscopic precipitation to a dense macroscopic precipitate, depending upon the composition of the tissue extract, the reagin titer of the syphilitic serum and the technique of the test. Thus precipitation tests may vary from a microscopic slide test to a macroscopic test tube reaction.

RÔLE OF INGREDIENTS.—The sensitivity and specificity of the precipitation reaction is largely determined by the following:

- (1) Fraction of total lipoids extracted from heart muscle.
- (2) Synergistic (or sensitizing) substances added to tissue extract.
- (3) Electrolyte concentration.
- (4) Lipoidal concentration.
- (5) Lipoidal-cholesterol ratio.
- (6) Alcohol-water ratio.

With the data available, it is impossible to evaluate the importance of each, nor can the relative interdependence of all or several be calculated with success. In order to simplify an ele-

mentary presentation of precipitation tests, the various ingredients and ratios of ingredients will be discussed as separate topics.

ANTIGENS.—The term “antigen” is a misnomer applied to the concentrated lipoidal tissue extracts used as a reagent in this test. However, in keeping with usage, the word antigen has been used in this chapter to designate tissue extracts. The antigen used in flocculation tests is composed of a portion of the lipoids of normal, rapidly dried, fresh heart muscle. Beef heart, freed of connective and fatty tissue, is generally used for this purpose. Any chemical compound in which lipoids are soluble and proteins are insoluble can be used as an extracting fluid. Several extracting fluids may be used as the total lipid content is not soluble in any one solvent. Further, solvents may be chosen to remove undesirable lipoidal fractions (as ether in preparation of Kahn antigen) and the dried residue extracted with other solvents for the “antigenic lipoids.” In general, the ether-soluble lipoids are characterized by their instability in water dispersions, while the ether-insoluble, alcohol-soluble lipoids give relatively stable suspensions.

The extracted lipoids may be separated into acetone-soluble and acetone-insoluble fractions. The acetone-insoluble fractions of the alcohol-soluble portion have given very satisfactory results in microscopic tests.

The character of the extract can again be modified by changing the temperature of the solvents during extractions. Each of the fractions of lipoids appears to differ slightly in chemical composition and activity but they grade into one another like colors of the spectrum. Hence any one fraction represents a series of lipoids. Chemical analysis has failed to reveal the structural formula or even the active compounds. The extractives appear as a light yellow to a dark brown waxy substance composed of lipoids of the cholesterol and lecithin series along with a larger undetermined fraction known as tissue extractives.

SYNERGISTIC SUBSTANCES.—Substances added to the alcoholic lipoidal extract which increase the sensitivity of the finished antigen are known as synergizing (or sensitizing) agents. These substances are compounds of known chemical purity, organic in nature and soluble in fat solvents but insoluble in water. The most important of this group is cholesterol. Cholesterol in alcoholic solution is precipitated by addition of water, giving crystals

of uniform dispersion for any set of standard conditions. The size of the cholesterol particles may be controlled by the relative volume of alcohol, concentration of cholesterol in the alcohol and the amount of water used as a dispersing fluid. Solution of lipoids gives suspensions of extreme variability, even when mixed under apparently identical conditions. The addition of cholesterol appears to stabilize the dispersion. Production of antigen particles of identical size under laboratory conditions is of great importance.

RÔLE OF CHOLESTEROL.—Eagle (1930) and others believe the rôle of cholesterol is purely physical in that it determines the size of the dispersed particle. The cholesterol forms the core and the antigenic lipoids cover it with a thin film. As the size of the particles increases, the total surface area is decreased, requiring less reagin to film the particle. This results in less reagin per unit volume of serum necessary to produce aggregates and automatically increases the sensitivity of the test according to Eagle. Other substances which may be substituted for cholesterol in precipitation tests are: solid sterols, lecithins, solid alcohols, balsam of Tolu, salicylic and benzoic acid.

In some of the new microscopic tests, the synergistic substance is dispersed before the lipoidal extract is added (Kline, Rosenthal, etc.). The finely precipitated lipoids are adsorbed on the surface of the cholesterol particles. Suspensions made in this manner have particles of greater consistency in size and behavior. The type of suspension differs from those made by precipitating the same amounts of lipoids and cholesterol from the same solution.

All of the synergistic substances used are insoluble, forming suspensions in the finished alcoholic-aqueous menstruum used as a reagent. Such suspensions follow the laws of physicochemical flocculation. This nonspecific chemical flocculation imposes limitations on the concentration which can be used to synergize (or sensitize) the lipoids.

These substances are without antigenic activity when used alone. The exact chemical and physical rôle of cholesterol or similar substances in the precipitation has not been determined.

WATER.—Water may be added separately as distilled water or as a solvent of some of the reagents, such as salt solution. The water serves as a dispersing medium for the particles of the anti-

gen and synergistic substances. It also affects the potential differences indirectly through its effect on the dissociation of electrolytes. Moreover, the reagin-filmed particles change from a liophylic to a liophobic surface interface resulting in precipitation. The water apparently has a physical rôle.

ELECTROLYTES.—The function of electrolytes in the precipitation test is identical with their action in the agglutination reaction. The reagin-lipoid complex acts as a denatured protein. This results in a lowered P.D. between the particles with drastic reduction in their repulsive forces. When such particles come in contact through agitation or other physical means, sticky aggregates are held together by the cohesive force.

From this it is evident that the concentration of electrolytes has an important bearing on the sensitivity of the test. Precipitation occurs with greater ease as the concentration of the electrolytes is increased. It also becomes more sensitive as the valence of the metallic ion is increased.

The various electrolyte concentrations which have been used in precipitation tests are listed in the following table:

Kahn	0.90	per cent sodium chloride
Kline	0.85	per cent sodium chloride
Meinicke	3.00	per cent sodium chloride and
	0.01	per cent sodium carbonate
Hinton	5.00	per cent sodium chloride and
	0.022	per cent salicylic acid
Rosenthal	0.40	per cent (only serum electrolytes)
Citochol	0.85	to 3.00 per cent sodium chloride
Mazzini	1.00	per cent salt concentration
		(buffered saline)

Deissler and Baker (1935) and others favor the increase in the electrolyte concentration as a means of increasing sensitivity and velocity of the reaction. From two to three per cent sodium chloride has given more satisfactory citochol reactions in the laboratories of the above workers and in many other laboratories.

ALCOHOL.—The lipoids are usually dissolved in alcohol or some similar lipid solvent. The alcohol has no rôle in the precipitation of the particles. It serves as a solvent for the lipoids until dispersion is accomplished from the solution by diluting it with water. It is an inert ingredient in the finished antigen since the changes in ionization constants are negligible.

Ratio of Components.—The ratio of concentrations between ingredients is of great importance. To appreciate this it would seem best to discuss separately the ratio between the various ingredients. It must be remembered, however, that they are definitely interdependent.

CONCENTRATION OF LIPOIDS.—The sensitivity of the reaction is dependent upon the number of milligrams of lipoids per cubic centimeter of extract. An optimum range is determined by empirical titration, using strongly positive, weakly positive, and negative sera. The preceding statement must be qualified, since it holds only in general for the lipoids within limited concentrations. The lipoidal solids obtained by the extraction methods used are crude mixtures of antigenic and nonantigenic lipoids, while the activity of the extract is due only to the antigenic lipoids. Until new methods of separation are devised, an exact quantitative ratio cannot be reached.

(1) Fraction of total lipoids extracted from heart muscle

CHOLESTEROL-LIPOID RATIO.—From the viewpoint of sensitivity of a newly prepared tissue extract, the cholesterol-lipoid ratio is of still greater importance. As the percentage of cholesterol in ratio to a given concentration of extractives increases, the precipitation value of this complex by human sera, especially syphilitic sera, increases to a maximum. Opinions differ concerning the cholesterol-lipoid ratio. Eagle (1931) believes the sensitivity of the antigen can be increased up to the cholesterol saturation point and recommends 0.8 per cent cholesterol and 0.6 per cent sitosterol in antigens. Levine (1932, 1933), on the other hand, believes these statements unwarranted. He contends that the cholesterol concentration required for maximum sensitivity depends upon the lipid concentration of the individual antigen. According to the Levine theory the sensitizing activity increases as the cholesterol concentration increases and after reaching a maximum (optimum lipid-cholesterol ratio), additional cholesterol will reduce the sensitivity of the antigen, following the so-called "colloidal type curve." It has been the experience in this laboratory that excessive cholesterol concentrations reduce the specificity of the test. Until more data are obtained, an intermediate view between the two extremes appears reasonable.

ALCOHOL-WATER RATIO—The ratio of water to alcohol is relatively unimportant. Sufficient alcohol must be used to carry in a stable solution the lipoidal substance. The water-alcohol ratio must be high enough to prevent solution of the lipoids in the finished suspension. After the suspension is prepared, the alcohol becomes inert so far as activity of the test is concerned.

The speed of "shaking" or "rotation" of the test is important since the velocity of precipitation is chiefly determined by this factor. It is assumed that the increased speed of precipitation is due in part to a more rapid filming of the antigen particles with reagin and partly to the increased number of contacts made between the filmed particles during mixing. The filmed particles have lost their high P.D. and are sticky; hence, once in contact, they tend to precipitate in clumps. The shaking principle was introduced into reagin tests by Gaechtgens and Holn and studied in detail by Kahn. According to the latter author, insufficient shaking results in negative reactions from sera low in reagin titer and excessive shaking leads to weak positive reactions in sera containing no reagin.

MISCELLANEOUS FACTORS.—Factors that also affect the size and shape of the dispersed particles are the speed of mixing, the ratio of masses of lipoids and water, the final alcohol or ether content, order of mixing, concentration of electrolytes, temperature of mixing and the age of the emulsion. In the precipitation test, as in the complement fixation test, the state of dispersion of the antigen is an important consideration.

Finished Antigen.—The finished antigen is a suspension of alcohol-soluble substance precipitated in an aqueous medium. According to Eagle the sensitivity increases, within certain limits, with the coarseness of the dispersed particles. Kline has modified this view, believing that sensitivity increases with size and shape only when all other factors of the test are held constant. Kline considers the shape equally important with surface area. However, on final analysis, as the size of the particle increases, the surface area is decreased per unit volume of suspension, which is the same as reducing the amount of antigen in the test. As the surface area decreases, the amount of reagin necessary for precipitation is correspondingly reduced.

The type of reaction depends upon the kind of antigen prepared. Macroscopic reactions require larger particles than microscopic reactions. The larger particles undergo a pseudoagglutination that is easily converted into larger aggregations in the presence of syphilitic serum. Such larger clumps give an easy end-point in reactions like the Kahn.

On the other hand, microscopic reactions must have antigens with finely divided particles. Such reactions are made on a microscopic slide, and the clumps are magnified from eighty to one hundred diameters. Minute clumps in the negative serum give pseudopositive reactions which interfere seriously with the test. Only slight clumping is necessary for a positive reaction with this magnification.

Choice of Test.—Numerous tests have been published, and although they differ in method, the mechanics of each is similar. The choice of a standard flocculation test awaits the accumulation of laboratory and clinical correlation over a long period of time. Until that time the choice of the flocculation test depends chiefly upon personal preference and individual success. Several of the most used of the flocculation tests will be briefly described.

Kahn Precipitation Reaction (1940).—The antigen consists of ether-insoluble, alcohol-soluble lipid fractions of beef heart with the addition of 0.6 per cent cholesterol. The antigen is standardized by a series of titrations.

SALT TITRATION.—This represents the minimum amount of 0.90 per cent saline necessary for dispersion of the antigen and that will also permit resolution on addition of 1 c.c. or less of isotonic saline after the emulsion has been shaken for three minutes with 0.15 c.c. of saline and incubated for fifteen minutes in a 37° C. water bath. This is known as the titer of the antigen and represents the amount of saline solution which must be added to 1 c.c. of lipid solution.

The procedure is best illustrated by the accompanying protocol of an actual titration (Tables XII, XIII). The protocol is modified after Kahn.

This gives a heavy white precipitate in each dilution vial. Three tubes containing respectively 0.05, 0.025, and 0.0125 c.c. of antigen are prepared for each dilution as in Table XIII.

TABLE XII
DISPERSION OF ANTIGEN

Number	DISPERSION OF ANTIGEN				
	1	2	3	4	5
Antigen (cholesterinized alcoholic)	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.
Salt sol. (0.90 per cent)	0.8 c.c.	0.9 c.c.	1.0 c.c.	1.1 c.c.	1.2 c.c.

Isotonic saline, 0.15 c.c., is added to each tube.

The tubes are shaken for three minutes and incubated in a 37° C. water bath for 15 minutes.

Salt solution is added after incubation.

In this titration, 1 c.c. of antigen plus 1 c.c. of isotonic saline is the titer. It is 1 c.c. of the antigen plus a minimum amount of salt solution giving a precipitate which dissolves in an excess of salt solution.

STANDARDIZATION OF ANTIGEN.—After the salt titer of the antigen has been found, comparison with the standard antigen is made as shown in Table XIV.

Antigens numbers 1 and 2 were freshly prepared antigens. In the comparison, antigen number 1 was almost identical in sensitivity and specificity with the standard antigen. Antigen number 2 was unfit for laboratory tests, since it was deficient in sensitivity. Except in the strongly positive serum, the reactions were consistently weaker. In the two very weakly positive sera, negative results were obtained.

An antigen, standardized as described, consists of coarse particles which, under conditions of the test, are dissolved by an excess of isotonic saline in the presence of normal serum but, when filmed with reagin of syphilitic serum, are insoluble and produce coarser macroscopic aggregates.

INACTIVATION.—The serum is inactivated by heating at 56° C. for thirty minutes. According to Kahn, sera low in reagin titer which give negative or weakly positive reactions before inactivation, give stronger reaction after heating. No satisfactory explanation has been offered.

SHAKING.—After mixing the serum and antigen, a three-minute shaking period has been found to give good precipitates with

TABLE XIII
TITRATION OF ANTIGEN

Number, as in Table XII	1		2		3		4		5	
Antigen (alcoholic-saline mixture)	0.05	0.025	0.0125	0.05	0.025	0.0125	0.05	0.025	0.0125	0.05 0.025 0.0125
Salt solution	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15 0.15 0.15
Shaking	Three minutes									
Incubation	Fifteen minutes at 37° C.									
Salt solution	1.0	0.5	0.5	1.0	0.5	0.5	1.0	0.5	0.5	1.0 0.5 0.5
Precipitate	+	+	+	+	+	+	-	-	-	- - -
Standard antigen dilution	1 + 1									

+ = Precipitate.

- = No precipitate.

TABLE XIV

Amount of anti- gen	STANDARD ANTIGEN			ANTIGEN NO. 1			ANTIGEN NO. 2		
	0.05	0.025	0.0125	0.05	0.025	0.0125	0.05	0.025	0.0125
Normal serum	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-
Syphilitic serum	++++	++++	++++	++++	++++	++++	++++	++++	++++
	+	++++	++++	+	++++	++++	-	+	++++
	-	+	++++	-	+	++++	-	-	-
	-	-	++++	-	+	++++	-	-	-
	++	++++	++++	+	++++	++++	-	+	++++

- = No flocculation.

+ = Very slight flocculation.

++ = Slight flocculation.

+++ = Moderate flocculation.

++++ = Complete flocculation.

syphilitic serum and no tendency to produce nonspecific precipitate.

TIME OF READING RESULTS.—The results are read immediately after the addition of salt solution and a second reading is made 15 minutes later. The six tube readings thus obtained are added and divided by 6, plus minus (\pm) readings being disregarded. He reports all readings of $\frac{3}{6}$ or less as negative, $\frac{4}{6}$ and $\frac{5}{6}$ as doubtful and readings more than $\frac{5}{6}$ as positive. For further details see Supplement No. 11, U. S. P. H. Service.

CONTROLS.—The test includes a positive serum, a negative serum, an antigen and a patient's serum control. Positive controls must have a precipitate while negative controls are free from a precipitate. The serum control is necessary to show that the serum under test will not give a pseudoprecipitate with saline solution only. The antigen control rules out any spontaneous precipitation of antigen by saline solution. To avoid false reactions, a weak ++++ or a +++ serum should be used as a positive control. This practice permits the discovery of slight mishaps in technique that would otherwise be unrecognized.

Choice of Kahn Test.—Three separate tests, besides his new verification test, are described by Kahn (1940). The routine test is employed in the general diagnosis of syphilis and as a routine test on admittance of cases to hospitals, etc. This is a three-

tube test requiring 0.45 c.c. of serum and three dilutions of antigen. Weakly positive sera react in the highest ratio of antigen-serum (1/12).

PRESUMPTIVE TEST.—A presumptive test, due to its high sensitivity, is used chiefly in the ruling out of syphilis. In rare cases it may be used as a check on treatment and the diagnosis of obscure manifestations of syphilis, but great care must be used in the interpretation of the results. In general, negative presumptive reactions can be accepted and positive reactions considered doubtful. The presumptive test requires 0.15 c.c. of serum and 0.01 c.c. of antigen.

QUANTITATIVE TEST.—The quantitative procedure is an eight-tube test, using dilutions of serum varying from undiluted to a 1:60 dilution. Each precipitating tube is assumed by definition to contain four reacting units. The titer of the serum is determined by substitution in the formula:

$$S = 4 \times D$$

where D is the highest reacting dilution and S is the titer. This quantitative procedure is of particular value in estimating the response of cases to treatment. It can also be used in the evaluation of new agents for treatment.

Spinal Fluid Procedure.—In spinal fluid procedure the standard Kahn antigen may be used but a new salt titration must be made. This is necessary because of the changed concentration of electrolytes resulting from the ammonium sulphate used in the test. The titration technique is similar to that of the routine Kahn salt titration from which it differs by increasing the amount of salt solution used to disperse the cholesterolized antigen (1.1 c.c. to 1.5 c.c. usually) and the addition of 0.15 c.c. of 5 per cent ammonium sulphate solution in place of 0.15 c.c. of sodium chloride.

The remainder of the test is conducted in a manner similar to the presumptive test or by using known dilutions of spinal fluid, like the quantitative procedure. One hundredth c.c. of antigen, prepared as indicated by the modified salt ammonium sulphate titration, is used in each tube.

Application of Quantitative Tests.—The quantitative Kahn, both with serum and spinal fluid, is applicable only when strong

positives are obtained by a routine test. Any attempt to apply them to weakly positive sera will result in negative results in all except the first tube.

The accompanying protocol (Table XV) represents the actual results of three quantitative serum Kahn reactions.

TABLE XV

Serum dilution in c.c.	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	Titer = $4 \times D$
Serum dilution	None	1:5	1:10	1:20	1:30	1:40	1:50	1:60	
Special antigen dil.	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	
Serum B	+	+	+	-	-	-	-	-	$4 \times 10 = 40$
Serum B ₁	+	-	-	-	-	-	-	-	$4 \times 1 = 4$
Serum L	+	+	+	+	+	+	+	-	$4 \times 50 = 200$
Normal serum	-	-	-	-	-	-	-	-	None
Saline only	-								

+ = precipitate.

- = no precipitate.

Serum B was a case at the beginning of treatment. Serum B₁ has received six weeks of treatment. Serum L was identified as a positive control. Normal serum and saline are necessary as negative controls. This is a composite table taken from laboratory data. The serum controls consisting of serum plus saline are not represented in the diagram.

Kahn's Verification Test.*—Kahn (1940, 1941) has studied the effect of temperature on the Kahn three tube flocculation test and compared the reactions obtained with normal reagin and syphilitic reagin. He finds that reagin in lower animals† and nonsyphilitic individuals gives stronger reactions at 1° C. than at 37° C. whereas the reverse is true with syphilitic reagin. The details of his test are given in his papers.

Description of Kline Test.—*Kline Microscopic Slide Precipitation Test.* The Kline test is a microscopic slide precipitation test for the detection of syphilitic reagin. It contains "microscopically visible lipid-coated cholesterol plates which clump after being acted upon by positive syphilitic serum or spinal fluid. There is probably a combination of the particle plus the reacting substance and a consequent surface alteration with a lessening of solubility." (Kline, 1931, 1940.)

*See pp. 434, 435 for a discussion of nonspecific reactions.

†For a review of literature on normal reagins see paper by Kemp, J. E., Fitzgerald, E. M., and Shepard, M.: *Am. J. Syph., Gonorr. and Ven. Dis.* 24: 537, 1940.

SENSITIVITY.—Kline claims for his later modifications an equal sensitivity and specificity with the Kolmer and the Kahn reactions. According to him, the diagnostic slide test is no less specific than other reliable tests for identification of syphilitic reagin.

AGREEMENT WITH OTHER TESTS.—The Kline test has an agreement of 95.2 per cent with complement fixation methods. The author claims an agreement of 94.9 per cent with clinical observations.

TECHNIQUE OF TESTS.—Serum for the test is obtained in the usual way. It is inactivated at 56° C. for 30 minutes in a water bath. The test is made on glass slides. A paraffin ring with an inside diameter of twelve millimeters is placed on a slide. It is most convenient to use slides, two inches by three inches, containing sixteen such rings.

PREPARATION OF ANTIGEN.—The antigen emulsion for the diagnostic procedure is prepared as follows:

Distilled water	0.85 c.c.
Cholesterol 1 per cent (absolute alcohol)	1.00 c.c.
Alcoholic extract of beef heart	0.10 c.c.
Isotonic saline	2.45 c.c.

The distilled water is placed in a small cork-finish bottle. Cholesterol solution is slowly added down the neck of the bottle and the mixture is rotated for twenty seconds. After addition of the antigen, the mixture is shaken for one minute, throwing the liquid from bottom to stopper each time. The saline solution is added and the shaking repeated, less vigorously than previously, for one minute. The antigen is "cured" for fifteen minutes in a 37° C. water bath.

TECHNIQUE OF TESTING.—With the end of the pipette touching the glass, 0.05 to 0.06 c.c. of inactivated serum is placed in the center of the paraffin ring. A drop of antigen containing between 0.0075 and 0.0085 c.c. is placed on top of the serum. The mixture is stirred with a toothpick. After four minutes' rotation, the test is completed.

READING TEST.—A microscopic examination, using a 10× ocular and a 16 mm. objective, is made. Distinct agglutination consisting of coarse clumps, is a strongly positive reaction. Finer clumps are interpreted as moderately or weakly positive reac-

tions. In this laboratory, very fine clumps have been considered as doubtful reactions and without diagnostic value.

Typical microreactions are shown in Figs. 20-23.

Fig. 20.

Fig. 21.

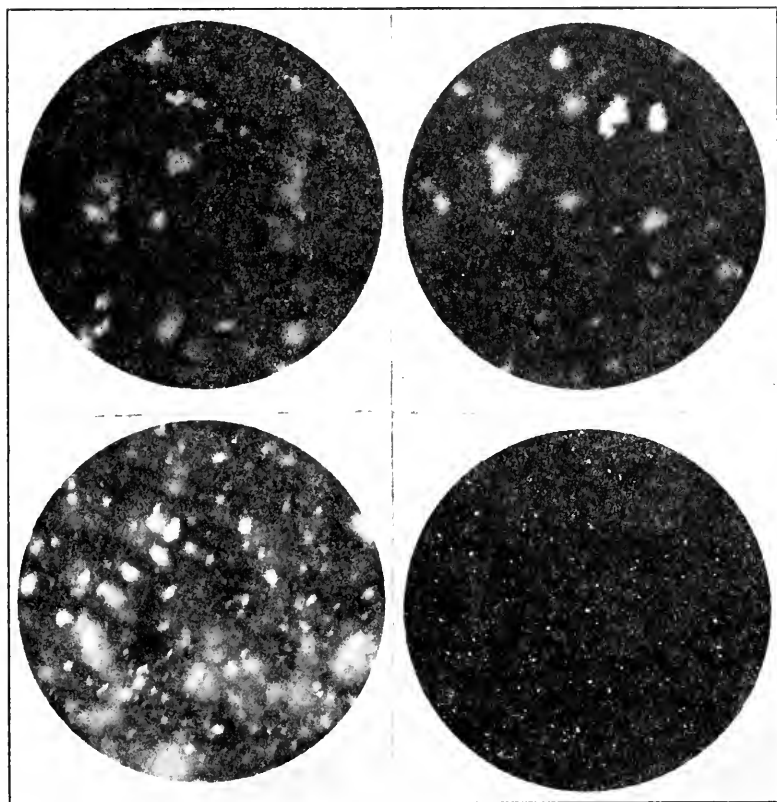


Fig. 22.

Fig. 23.

Fig. 20.—Photomicrograph of a strongly positive Kline test. (Dark-field illumination.)

Fig. 21.—Photomicrograph of a moderately positive Kline test. (Dark-field illumination.)

Fig. 22.—Photomicrograph of a weakly positive Kline test. (Dark-field illumination.)

Fig. 23.—Photomicrograph of a negative Kline test. (Dark-field illumination.)

The strongly positive reaction (Fig. 20) has large microscopic clumps. As the reagin titer decreases, the clumps become smaller (Figs. 21 and 22). A typical negative reaction (Fig. 23) has a number of needlelike crystals equally distributed over the field.

A strongly positive serum usually produces clumps of sufficient size for macroscopic diagnosis. This leads to the omission of the microscopic examination—a practice to be condemned. Weakly positive reactions are easily missed when such a practice is followed.

It is also recommended that a positive and a negative serum control be included in each series of tests (on each side). Antigen (0.0070 c.c.) plus saline (0.05 c.c.) is also included in this laboratory as an additional control.

In addition to the routine diagnostic test described above, Kline has an elimination test, comparable in purpose to the presumptive test of Kahn. This test differs in the preparation of the antigen. It is as follows:

Distilled water	0.85 c.c.
Cholesterol 1 per cent ab. alcohol	1.0 c.c.
Antigen	0.1 c.c.
Sodium chloride, C.P., 0.85 per cent	2.45 c.c.

ELIMINATION TEST.—The emulsion is prepared as for the diagnostic test. After being incubated in a water bath at 56° C. for 15 minutes, it is centrifuged for fifteen minutes (eighth setting rheostat, centrifuge size 1, type S. B.). The supernatant fluid is removed and the sides of the tube are dried with blotting paper. The sediment is resuspended in 1.0 c.c. of isotonic salt solution. The technique for the test is like that of the diagnostic reaction. More details pertaining to the Kline tests are given in Supplement No. 11.

USE OF ELIMINATION TEST.—The elimination test is useful in cases in which it is desired to rule out syphilis as a possible complication. It has been successfully used in testing blood donors, in health examinations, and in routine examination of patients being admitted to institutions or hospitals for other than venereal treatment.

SOURCES OF ERROR.—A common source of difficulty resides in the cholesterol solution. To give satisfactory results, the cholesterol must be weighed accurately. The error should not exceed 0.005 grams per gram of cholesterol. The alcohol used in preparing the solution should be free from traces of water. Such alcohol will not color anhydrous copper sulphate. This reagent rapidly absorbs

moisture from the air after preparation. Thus fresh cholesterol reagents should be prepared frequently. The sensitivity of the emulsion is more dependent upon the cholesterol content than on the antigen content. The antigen extract can be varied over a range of 400 per cent while the cholesterol content has a range of 60 per cent in satisfactory emulsions. (Kline, 1931.)

Dirty glassware and slides may result in spontaneous agglutination.

Principle of the Meinicke Reaction.—In the Meinicke reaction the reagin in the syphilitic serum disturbs the colloidal balance of beef (or horse*) heart lipoids dispersed in three and one-half per cent sodium chloride solution. The result is a microscopic to a macroscopic flocculation, depending upon which of the several modifications of Meinicke is followed. Negative serum, lacking reagin, does not change its uniform turbidity. The precipitate is composed of a lipoidal fraction and balsam of Tolu filmed with reagin and a denatured globulin fraction. This reaction, according to Meinicke, is a typical lipoid-binding reaction. It is equivalent to agglutination or complement fixation in specificity; and, with an appropriate antigen, can be applied to the diagnosis of any infectious disease or differentiation of a specific protein. It is Meinicke's conception that the antigen-antibody complex binds lipoids (1926).

SPECIFICITY.—Meinicke (1917, 1918, 1926-27), Jantzen (1921) and Epstein and Paul (1921) claim equal specificity with the complement fixation methods in general use. They believe this flocculation method is slightly more sensitive in general syphilis and decidedly more sensitive for the serological diagnosis of lues congenita.

ANTIGEN.—The antigen is an ether-insoluble, alcohol-soluble extract of beef heart. To this extract is added 1.4 per cent balsam of Tolu and 0.01 per cent Victoria blue. The antigen is titrated with weakly positive and negative serum by an arbitrary method. (Meinicke, 1934.)

MIXING OF ANTIGEN.—Reagents in the test are antigen extract and three and one-half per cent sodium chloride solution. A

*In the earlier modification of the Meinicke reaction the antigen was prepared from horse heart lipoids, but in his recent modification (1934) he uses beef heart lipoids.

dispersion is prepared by heating the ingredients to 55° C. in a water bath and pouring the saline solution into the antigen. Thorough mixing is accomplished by pouring back and forth ten times. The diluted antigen is ready for immediate use.

SERUM.—The serum is obtained in the usual manner. It *must not* be inactivated.

TECHNIQUE OF THE TEST.—Place 0.2 c.c. of active (raw) serum in a small test tube of the Kahn type and add 0.5 c.c. of freshly prepared suspension (M.K.R.II suspension). Mix the ingredients thoroughly and incubate at room temperature.

INTERPRETATION OF RESULTS.—According to Meinicke the test can be read by any of the four methods outlined in the following discussion. It has been our experience that the macroscopic flocculation method is the safest procedure to follow when testing weakly positive sera. In our hands the test has no distinct advantage over the Kahn and Kline tests. The four methods of reading the test are:

1. Macroscopic flocculation reaction: The flocculation is read like an agglutination reaction after one and one-half hours of incubation. Using a hand lens distinct clumps are present in positive serum which are absent in a negative reaction.

2. Microscopic reaction: A few drops of the liquid are removed from the serum-suspension mixture when it is set up and placed in a paraffin ring on a slide. This is incubated for one hour in a moist chamber.

When magnified sixty diameters, clumps indicate a positive reaction which are absent in the negative serum.

3. Clarification reaction: Incubate tubes overnight at room temperature. Negative reaction remains uniformly turbid. The degree of clearing of the supernatant liquid indicates the strength of the positive reaction with a strong positive reaction becoming completely clear.

4. Centrifugal technique: The tubes are centrifuged at low speed for ten minutes immediately after setting up the test. The speed can be determined by trial for each centrifuge, which will exclude nonspecific reactions (Dombrowsky, 1933). All supernatant liquid is poured out and the tubes are inverted for half an

hour in a Wassermann rack. Unaltered sediment is positive, while the reaction is negative if the sediment has run down the side of the tube.

Hinton Test.—HINTON ANTIGEN.—This test is known as the second modification of Hinton (1930, 1931, 1940). The reaction is a *macroscopic* flocculation test for reagin. The lipoid extract consists of the ether-insoluble, alcohol-soluble lipoids of beef heart with 0.4 per cent cholesterol added.

GLYCERATED INDICATOR SOLUTION.—The antigen used in making the test is called a "glycerinated indicator solution." A one hundred c.c. Erlenmeyer flask that has a ridge across the bottom dividing it into two equal compartments is used.

The reagents used in preparing the antigen are:

1. Cholesterolized beef heart extract.
2. Salt-salicylic acid mixture containing 5 per cent sodium chloride and 0.00222 per cent salicylic acid.
3. Fifty per cent neutral glycerol (glycerol diluted with distilled water).

TECHNIQUE OF TEST.—One cubic centimeter of cholesterolized antigen is placed on one side of the ridge and 0.8 c.c. of the salt-salicylic acid mixture on the other side. The reagents are mixed by rotating for one minute and allowed to stand at room temperature for five minutes. Thirteen and two tenths c.c. more of salt-salicylic acid mixture are added to the suspension in the flask and after thoroughly mixing, 15 c.c. of the 50 per cent glycerol solution are added. This makes a 1:30 dilution of the original beef heart extract. The indicator solution keeps one week at ice box temperature.

Serum is inactivated at 55° C. for thirty minutes. Five-tenths c.c. each of serum and indicator are pipetted into a test tube (10 mm. × 100 mm.). The rack is inclined at an angle of 45° and shaken in such a manner that the liquid travels halfway up the side of the tube with each forward thrust. The shaking period is three minutes.

The tubes are incubated in a 37° C. water bath for sixteen hours.

In the positive reaction there is a ring or band of coarse granules at the top of the tube, accompanied by complete clearing of

the fluid. Gentle shaking causes the particles to disperse and appear as a definite precipitate throughout the liquid.

In the negative reaction there is no band and no precipitate. The liquid usually has a faint opalescence.

Hinton claims greater speed in testing and equal specificity with the Wassermann reaction.

Although salicylic acid and glycerol have been added apparently as preservatives, the occasional growth of bacteria in the mixture, when sterility is not accomplished, is a decided disadvantage. Such contamination in tubes may be easily mistaken for positive reactions.

Citochol Reaction.—The citochol reaction of Sachs and Witebsky appears to be gaining popularity in America. Many laboratories find it to possess a high degree of specificity and sensitivity checking closely with other precipitation and complement fixation tests (Beintema, 1934, Stern, 1933, Deissler and Baker, 1935 and others). This reaction is similar in principle to the reactions discussed, bearing a closer resemblance to the Kahn and Meinicke. It differs from the newer precipitation tests in that the authors (Sachs and Witebsky) consider the preparation of the antigen comparatively unimportant. The antigen is a concentration of the Sachs-Georgi antigen used in the slower citochol reaction. Cholesterol in concentrations of three- to six-tenths per cent is used as a synergizing agent, being dissolved in the concentrated alcoholic lipoidal extract.

ANTIGEN.—The lipoidal extract is prepared by extracting one part of moist beef heart with five parts of alcohol. The extract is evaporated to dryness and dissolved in three volumes of alcohol. To the finished extract is added 0.3 to 0.6 per cent cholesterol. A suspension is made by mixing one part of cholesterolized extract with two parts of saline solution and after standing five minutes, adding nine parts of saline solution, making the final antigen dilution 1:12. Nine-tenths per cent sodium chloride solution was originally used as a diluting fluid but the test is more sensitive if 2.5 per cent sodium chloride is substituted (Deissler and Baker, 1935).

SERUM.—The serum is inactivated by heating at 55° C. for thirty minutes. Inactivation in quantities of less than half a

cubic centimeter gives unsatisfactory results and one cubic centimeter quantities should be used for inactivation whenever possible.

TEST.—A mixture of two-tenths cubic centimeter of suspension and an equal volume of inactivated serum is shaken three minutes in a Kahn shaking machine. One c.c. of saline solution is added. Particles can be observed in the strong positive tests, while the doubtful positives can be read by means of the agglutinoscope. After standing twenty-four hours, it can be read as a clarification reaction. An alternative method of reading is microscopic examination (eighty diameters magnification) of the mixture for clumping before addition of the final saline solution.

Advocates of the test claim specificity and sensitivity equal to the Kahn over which it has no apparent advantage. The disadvantage of the test is the lack of quantitative data as to reagin titer of the positive serum.

Bruck's Nitric Acid Reaction.—Bruck (1917) precipitated serum with nitric acid. The precipitate formed in syphilitic serum was less soluble in dilute nitric acid than that of normal serum. Its agreement with complement fixation methods was about 70 per cent. The reaction appears to have little practical application.

Formol Reaction.—Gaté and Papacostas (1920) reported that two drops of commercial formalin added to syphilitic serum produced a solid gel in 24 hours. Normal serum was unchanged. In their paper, they claim 85 per cent agreement with complement fixation test. Ecker (1921), Pauzat (1920) and others report the test as giving inconsistent results.

Sachs-Georgi Reaction.—Sachs-Georgi (1920) reaction is a flocculation test using a cholesterolized lipoid antigen from beef heart. A precipitate forms in syphilitic serum which does not appear in normal serum under the conditions of testing.

Vernes.—Vernes' reaction is based on the difference in turbidity produced by colloidal substances. Colloidal ferric hydroxide, along with other inorganic colloids, will produce greater flocculation in syphilitic than in normal serum.

Eagle Flocculation Test.—Eagle (1932, 1940) has described a new flocculation test for syphilis claiming a greater sensitivity than

existing tests. This increased sensitivity is secured by addition of both cholesterol and sitosterol to the antigen and a modification in manipulation.

Rosenthal Test.—Rosenthal (1929) has reported a new sensitive flocculation test. This increased sensitivity is accomplished through an increase in the cholesterol content of the reacting mixture. The antigen is essentially a Kahn antigen without the addition of cholesterol. Equal parts of cholesterol solution (2 per cent in acetone) and antigen are mixed. Fifty milligrams of methylene blue are added to 10 c.c. of the mixed reagent. This dye stains the particles. A drop of the stained antigen is added to four drops of inactivated serum. Examination is made with a magnification of 80 to 100 diameter. Clumping occurs in syphilitic serum.

Mazzini Microflocculation Test.—In the Mazzini (1939) test the antigen contains acetone insoluble, alcohol soluble lipoids from both dehydrated beef powder and powdered egg yolk, and also cholesterol and buffered saline. The student is referred to Mazzini's publication for a description of the test. According to Ratcliffe (1940) the Mazzini test is superior to "certain other tests in common use."

It has been impossible to discuss in the space of one chapter all of the flocculation reactions used in the serological diagnosis of syphilis. Outstanding types of reactions have been cited as examples to establish general principles. This policy has, perhaps, led to the omission of many valuable and efficient procedures.

Importance of Using Both Complement Fixation and Precipitation Tests.—None of the methods described will replace the complement fixation methods entirely. There are sera which will give positive flocculation tests and negative complement fixation tests. The reverse is also true. It would seem better practice to use both the flocculation and complement fixation tests as routine procedures. It will greatly increase the accuracy of the results and reduce the number of false reactions to a minimum.

Hypersensitive Tests of Doubtful Value.—The use of hypersensitive tests, either complement fixation or flocculation, is condemned by Levine (1933). Transitions from primary to early and to later secondary stages are associated with definite changes

in the intensity of the serological reactions. (Moore and Kemp, 1926.) For this reason the sensitivity of any reaction should be correlated with a great number of clinical cases before acceptance.

In considering the choice of a precipitation test, the following points should be considered. The test should be:

1. Highly specific.
2. Sensitive enough to detect very small amounts of syphilitic reagin.
3. Sufficiently quantitative to show changes in reagin during antisyphilitic therapy.
4. Decisive in results giving a very small zone of doubtful reactions.
5. Of simple technique, adequately described, giving consistent results in various laboratories and not requiring special training other than that of a qualified technician.

The precipitation method and complement fixation method are described as an aid to clinical diagnosis but not a substitute for it. They should be used accordingly.

Several tests should be made on each serum. Such a procedure is of distinct value both to the laboratory technician making the test and the physician. In this laboratory a quantitative Kolmer complement fixation test, a routine Kahn, and a diagnostic Kline are made on each serum. Irregular reactions can be investigated carefully with such a routine. The time required for the multiple-type tests is little more than that required for one type of test.

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CHAPTER XXV

HYPERSENSITIVENESS

Anaphylaxis

Discovery of Anaphylaxis.—In 1902 Richet and Portier undertook the study of the toxic substances present in the tentacles of *Actinaria* with an idea of comparing this substance with a similar one present in *Physalia* found in the South Seas. They prepared glycerin extracts and injected graded doses into dogs in order to determine the toxic dose. The animals which recovered were saved for further experiments. When they attempted to use these animals in later experiments, they discovered that the dogs had become intensively sensitive to the poison. Richet says that, "The most typical experiment, that in which the result was indisputable, was carried out on a particularly healthy dog. It was given at first 0.1 c.c. of the glycerin extract without becoming ill; twenty-two days later, as it was in perfect health, I gave a second injection of the same amount. In a few seconds it was extremely ill, breathing became distressful and panting; it could scarcely drag itself along, lay on its side, was seized with diarrhea, vomited blood, and died in twenty-five minutes."

Early Studies of Anaphylaxis.—In reviewing the literature Richet (1913) and also Doerr (1909) state that in 1839 Magendie observed that one injection of a nontoxic dose of albumin rendered rabbits, after several days had elapsed, sensitive to a similar dose of the same material. In 1890 Koch reported that tuberculous animals were hypersensitive to normally nontoxic doses of tuberculin. Flexner in 1894 found that rabbits that had survived a first injection of dog serum succumbed when given a similar or even smaller dose several days later. Von Behring (1893) noted, in his studies of diphtheria toxin, that one injection of toxin rendered guinea pigs more intensely sensitive to diphtheria toxin. Arloing and Courmont (1894) found that individuals receiving several injections of donkey serum showed definite reactions to later injections of donkey serum.

RICHET AND PORTIER'S WORK.—Richet and Héricourt (1898) observed that when dogs were injected with eel serum, they became sensitive to this material. Richet (1913) says that neither he and Héricourt, nor any of the others who had in the past observed sensitiveness, appreciated the significance of their observations. It was not until he and Portier rediscovered and investigated the phenomenon in 1902 that its significance was appreciated. They named this particular kind of hypersensitiveness "anaphylaxis" since in their opinion it was the opposite of "phylaxis" or protection.

ARTHUS PHENOMENON.—The following year (1903) Arthus rendered rabbits hypersensitive to horse serum. He injected the antigen subcutaneously into the animals at definite intervals and observed that whereas the material comprising the first three injections was rapidly absorbed, such was not true when later injections were made. Following these injections the material was not absorbed and there developed at the site of inoculation definite induration and not infrequently gangrene. Arthus also observed general systemic reactions in rabbits that had received several subcutaneous injections and finally an intravenous injection of horse serum. He noted also that the reaction is specific since he found that animals sensitized to horse serum are not sensitive to milk and, conversely, animals sensitive to milk are not sensitive to horse serum.

THE THEOBALD SMITH PHENOMENON.—In America, Theobald Smith (1903) observed anaphylaxis in guinea pigs used to test diphtheria antitoxin. He advised Ehrlich of his observations and the latter had Otto investigate the phenomenon. They were unaware of the earlier work of Richet and Portier.

ROSENAU'S AND ANDERSON'S STUDIES.—Coincident with Otto's investigation, Rosenau and Anderson (1906) reported extensive studies of anaphylaxis. The results of both investigations were published almost simultaneously. Only a few facts of fundamental importance relative to anaphylaxis in animals have been added since these studies appeared.

They determined that one injection of a nontoxic dose of horse serum will render guinea pigs hypersensitive to a second injection of the antigen provided an interval of almost ten days is allowed

to intervene between the injections. While both injections may be given subcutaneously, intraperitoneally, or intravenously, the second, i.e., the one to elicit shock, is given as a rule either intraperitoneally or directly into the blood stream, since it is difficult to produce severe reactions and death with even large doses administered subcutaneously.

They found, also, that the reaction is specific, that the specific hypersensitiveness is transmissible from the mother to the offspring, that the state of hypersusceptibility may be induced by any antigen and persists, when once acquired, for a long time. They observed that the animals which recovered from anaphylactic shock are immediately refractory to another injection of antigen. A sensitive animal can be made refractory by injecting, usually subcutaneously, a dose of antigen too small to cause symptoms of shock. When the animal is thus made refractory to the antigen, it is said to be "desensitized" and the process of rendering it refractory is called "desensitization." The duration of the refractory state, when once established, varies with the species of animal used in the experiment. In guinea pigs it is said to last two or more weeks, while in rabbits it is very short. Our own experience has indicated that rabbits may become sensitive again within twelve hours.

DISCOVERY OF PASSIVE SENSITIZATION.—In 1907 Nicolle found that when blood from a rabbit that had been sensitized with horse serum was injected into a normal rabbit, the latter became sensitive within the next twenty-four hours. The process of transferring to a normal animal specific hypersensitiveness to an antigen by injecting blood taken from a sensitized animal is called *passive sensitization*.

The information presented thus far enables one to offer a few definitions that may enable the student to better understand the subject.

Sensitizing Substance.—Any true antigen as defined in previous chapters can be used as the sensitizing agent. This suggests what is now well established, that sensitization depends upon the development of antibodies.

Landsteiner and Jacobs (1936) report success in producing anaphylaxis in guinea pigs sensitized with p-chlorobenzoyl chloride

and arsphenamine respectively. Landsteiner and Chase (1937) report upon anaphylaxis in animals induced by picryl chloride and 2:4 dinitrochlorobenzene. In their opinion their results offer strong evidence that antigenic conjugates are formed following the application of substances of simple chemical constitution. That is to say that the simple chemical substances used probably formed conjugates with the animals' own proteins, forming new antigens. The presence of these new antigens resulted in sensitization.

Sensitizing Dose of Antigen.—The initial or sensitizing dose or doses of antigen may be introduced by injecting the material into the tissues, blood stream or body cavity. Sensitization through the respiratory and alimentary tracts has also been demonstrated. This indicates that under certain conditions sufficient antigen may be absorbed through either the respiratory or intestinal epithelium to bring about sensitization. Rosenau and Anderson found that the sensitization of one guinea pig was accomplished by the injection of 0.000,001 c.e. (one millionth) of horse serum. Coea (1927) says that guinea pigs have been actively sensitized with as little as 0.000,000,5 gram of crystalline egg albumen. He further states that it requires from 1,000 to 10,000 times as much antigen to sensitize rabbits as guinea pigs.

Incubation Period in Active Sensitization.—The incubation period in active sensitization is the period of time between the administration of the sensitizing dose and the development of hypersensitiveness. In guinea pigs this varies from five to ten days. It has been observed that the smaller the sensitizing dose employed the longer the incubation period. Coea (1927) says that it may be as long as nineteen to twenty-five days where 0.000,1 to 0.000,01 c.e. of ox serum is used. Rabbits and dogs are more difficult to sensitize than guinea pigs. Auer (1915) recommends that rabbits be given four to eight injections of antigen subcutaneously four to eight days apart. He quotes Arthus as finding the incubation period to be from eight to fifteen days after the last injection.

Bally (1929) working in this laboratory succeeded in sensitizing rabbits to horse serum by administering 0.5 c.e. per kilogram of body weight subcutaneously followed in forty-eight hours by an intravenous injection of the same amount. He gave the shock-

ing dose eighteen or more days later and found all of the rabbits actively sensitized. The technique recommended by Manwaring is, perhaps, the best for sensitizing dogs. Bally employed it in his work in rabbits mentioned above. Using this method, Sherwood and Stoland (1929) found the incubation period in dogs to vary from nine to fourteen days after the second injection of horse serum.

Duration of the Hypersensitive State.—The duration of hypersensitiveness in guinea pigs probably persists throughout the lifetime of the animal. Coea (1927) states that rabbits remain sensitive, following a single injection of antigen, for about three weeks. Bally (1929) observed the hypersensitive state to persist in rabbits as long as seventy-seven days after two sensitizing doses of horse serum.

Shocking Dose of Antigen.—The shocking dose of antigen is the dose of antigen administered to produce symptoms of anaphylaxis. When given subcutaneously, large doses must be employed. The best results are obtained when it is administered intraperitoneally or intravenously, although the subdural and intracerebral routes may be employed. The shocking dose should be considerably larger than the minimum sensitizing dose of the antigen.

While it is necessary to employ a complete antigen to sensitize an animal, it has been shown by Tomesik and Kurotchkin (1928), and by Avery and Tillett (1929) that anaphylactic shock can be produced in guinea pigs passively sensitized to specific bacteria by injecting the carbohydrate specific for the bacteria in question. In this work it has been found important to use antibacterial serum from rabbits since immune serum from horses yields negative results.

Goodner and Horsfall (1937) inferred from their studies on anaphylactic shock in guinea pigs rendered passively sensitive to pneumococcal capsular polysaccharide that the ratio of antigen to antibody is very important. If the proportion of carbohydrate is slightly in excess of the amount necessary to satisfy the available antibody, a fatal response is possible. If the antigen is present in excessively large proportion, the result will be negative as is the case when the amount of antigen is too small.

Passive Sensitization.—Passive sensitization is the transference of hypersensitiveness to a normal animal by injecting blood containing specific antibodies obtained from an actively sensitized animal. To be successful it is necessary that blood be obtained from the donor (sensitized animal) at a time when antibodies are present in the general circulation; that a sufficient amount be employed to convey hypersensitiveness (this depends upon the concentration of antibodies in the blood); and that a normal animal be chosen as a recipient whose tissues can combine with the donor's antibodies and become hypersensitive to the specific antigen.

While passive sensitization is best accomplished where the donor and recipient are of the same species, it is well known that blood or serum from different species may be employed. Doerr (1909) and Sherwood and Downs (1928) observed that the sensitizing antibodies of one species may in some instances be incapable of sensitizing another species.

Sherwood and Stoland (1930) found a variation in the recipients of the same species. They transfused four small, healthy dogs of about the same age and weight with equal amounts of defibrinated blood obtained from a large sensitized dog. One of the recipients was rendered extremely sensitive, one was refractory (not sensitized) and two were moderately sensitive as judged by the drop in arterial blood pressure.

The duration of hypersensitiveness produced by passive sensitization varies in different species. It persists longer when homologous immune blood or serum is used than when the antibodies are obtained from a different species. Coca (1927) says that when a normal guinea pig is sensitized by immune serum from another guinea pig, the former may remain sensitive for sixty to seventy days. If heterologous immune serum is used, the hypersensitivity persists for about ten days only. Coca quotes Friedmann (1909) as saying that passive hypersensitiveness in the rabbit disappears within twenty-four hours, and Richet (1908) as finding that it persists in the dog for twenty days.

In connection with passive hypersensitiveness, various species exhibit interesting differences in the time between the receipt of antibodies and the development of hypersensitiveness to the

specific antigen. Manwaring (1910) and Scott (1910-11) report that dogs and rabbits respectively become sensitive immediately. In the case of guinea pigs, it is well established that at least four to six hours must intervene before hypersensitiveness can be demonstrated.* It is customary to test animals twenty-four to forty-eight hours after they receive immune serum.

Desensitization.—An animal can be desensitized usually by one injection of an amount of antigen that either produces mild shock or produces no symptoms. Large amounts can be employed where the antigen is administered subcutaneously and in fractional doses. The phenomenon of desensitization is important in the study of anaphylaxis in the experimental animal or when the “Dale” reaction is being employed. It should be remembered that practically all native proteins such as egg white, blood serum, etc., are more or less toxic for laboratory animals. This is true also for the uterine horns of virgin guinea pigs used in the Dale technique. An animal or the excised uterine horns become desensitized following an anaphylactic response. While the systemic symptoms of toxic reactions and anaphylactic shock can be differentiated as a rule, the uterine horn response to toxic doses of protein is identical in appearance with the anaphylactic response. To prove that the response of a uterine horn is anaphylactic rather than toxic, it is customary to repeat the shock dose. This should produce no reaction if desensitization has occurred. If a reaction occurs, it suggests that the original response might have been toxic rather than anaphylactic. The failure to observe such a criterion, described by Dale, has led to much confusion in the literature. Silva (1941) has suggested that trypsin may play an indirect role in anaphylaxis.

The Refractory State.—Animals supposedly rendered sensitive may not develop symptoms following the injection of a shock dose of antigen for several reasons which may be enumerated as follows:

1. They may not have been made sensitive either by the injection of antigen or by passive transfer. Antigens vary in their sensitizing capacity, and animals vary in their capacity to respond and become sensitive. Crystalline egg albumen is superior to native egg white as an antigen.

*Bronfenbrenner maintains that immediate sensitization occurs if homologous rather than heterologous immune serum is used.

2. Animals are refractory following shock, or the injection of desensitizing doses of antigen. This is due probably to the fact that the antibodies attached to the tissue cells are exhausted or saturated with antigen. This refractory state is called "*anti-anaphylaxis*" by Besredka and Steinhardt.

3. When the antibody content of the blood stream is high, the animal may not develop symptoms following the injection of antigen. It is thought that this is due to the union of antigen and antibody in the blood stream and that, for this reason, the former is not available to unite with antibody bound by the tissues. This is called "*masked anaphylaxis*."

4. Animals may be protected by drugs. The literature on this subject is reviewed by Auer (1915). According to him, Friedberger and Hartoch protected guinea pigs by injecting about 1 c.c. of saturated sodium chloride intravenously before the antigen was administered. Biedl and Kraus prevented anaphylaxis in dogs by injecting barium chloride. They also state that animals recovering from peptone shock are refractory to anaphylactic shock. Likewise, atropine, adrenalin, chloral hydrate, and even ether when administered to guinea pigs tend to reduce the severity of the symptoms and prevent death in a fairly high percentage of cases. They have no effect upon the antigen-antibody reaction, but affect tissue mechanisms only.

5. Bronfenbrenner's (1914, 1915) experimental results suggest that a refractory state may occur when the antitryptic index is high.

While the symptoms of acute or protracted anaphylactic shock exhibited by all members of any one species are the same, it is interesting to note that because of anatomical and physiological differences in species each exhibits its own characteristic symptoms. These, along with a number of additional facts concerning anaphylaxis, will be presented in the following brief discussion.

Anaphylaxis in the Guinea Pig.—ACUTE SHOCK.—Symptoms of acute shock in the guinea pig develop within one or two minutes following the intravenous or intracardial or intraperitoneal injection of an adequate amount of antigen. The animal is quiet for a few moments, then is restless and becomes excited, there is a roughening of the hair, the pig sneezes, rubs its nose or ears

and may discharge urine and feces, the movements of the alar nasi and of the respiratory muscles indicate difficulty in breathing. The animal usually coughs, jumps, staggers, falls over, and makes rhythmical movements of the extremities and violent movements of the muscles of respiration. It dies of asphyxia. If the thorax is opened immediately, it will be observed that the lungs are distended and cannot be made to collapse. The heart will continue to beat for some time although Auer and Lewis showed that heart block is present. They observed two or three auricular to each ventricular beat. The principal pathological finding is the distention of the lungs with air. This was first observed by Gay and Southard. Auer and Lewis conclude that this is the immediate cause of death. In their opinion it results from a tetanic contraction of the smooth musculature of the bronchioles, producing stenosis. According to Schultz and Jordan, this is localized in the secondary and tertiary bronchi. Auer says that they claim: "the tetanic contraction of the muscle coats folds the mucous membrane of this area into a plug which occludes the lumen and thus brings about asphyxia."

PROTRACTED OR SUBACUTE SHOCK IN THE GUINEA PIG.—When the pig dies following prolonged or protracted shock, the lungs are only partly distended. While this condition may be a contributing factor to death, it is possible that a drop in blood pressure is also important. Another fairly constant finding in protracted shock is a marked fall in body temperature. This is regarded as a characteristic symptom by Braun, Friedberger, and others. In addition to these findings, a prolongation of the clotting time of the blood, a definite leucopenia, and a diminution in complement titer have been reported.

THE DALE PHENOMENON.—In 1910 Schultz reported that if one removes short segments of the small intestine from a sensitized guinea pig and suspends them in a bath of Locke's or Ringer's solution kept at body temperature, they will undergo specific tetanic contraction when the homologous antigen is added to the bath. Dale (1913) studied this phenomenon and found that although Schultz used toxic doses of antigen, nevertheless, a specific contraction can be demonstrated when nontoxic doses are employed. He suggests that excised uterine horns from young virgin sensitized guinea pigs be employed instead of the loops

or segments of small intestine. The contractions of the uterine horn are recorded by means of a light heart lever attached at one end by means of a thread to the upper end of the uterine horn, while the other end of the heart lever produces a kymographic tracing on smoked paper. As previously mentioned, it is necessary to demonstrate desensitization to differentiate between toxic and anaphylactic reactions. The latter phenomenon is called quite frequently "the smooth muscle reaction of Dale." Stoland and Sherwood (1923) showed that when adequate doses of atropine are added to the bath, specific reactions are prevented. In their opinion Tyrode's solution containing dextrose and half the usual amount of calcium is superior to either Locke's or Ringer's solution.

Anaphylactic Shock in the Rabbit.—The first extensive studies of anaphylaxis in rabbits were carried out by Arthus (1903). When a sensitized rabbit is given an adequate intravenous injection of antigen, it may develop symptoms before the injection is completed or it may remain quiet for one or two minutes and then become excited, run aimlessly about, and die suddenly with its head retracted and eyes in exophthalmus. This is *acute fatal anaphylactic shock* in the rabbit. The cause of death has been studied by Auer, Coca, Airila and others. They have apparently shown that it is due to right heart failure caused by an increase in resistance to blood flow through the pulmonary circuit. The lungs are observed to be completely collapsed when the chest is opened. Arthus observed a prolongation of the clotting time of the blood. Bally (1929), working in our laboratory, carried out extensive physiological studies of anaphylaxis in rabbits. He summarizes some of his results as follows:

"1. The characteristic blood pressure response is an increased pressure followed by a decreased pressure which slowly returns to normal in the case of recovery.

"2. There is a peripheral circulatory response shown by the 'blanching reaction' of the ear to be much more pronounced than in either peptone or histamine shock.

"3. There is a tachycardia during the blood pressure increase which gives way to a bradycardia during the blood pressure fall and persists after the blood pressure has reestablished itself.

These phenomena resemble the various ones produced by histamine more closely than peptone at similar points on the blood pressure curve.

“4. A definite increase in coagulation time develops soon after the shocking dose of antigen is administered.

“5. A marked drop in the precipitin content of the blood occurs after intoxication, but there is not a complete loss of precipitins.

“6. A markedly engorged right heart with the lungs well collapsed in rabbits dying of shock is observed.

“7. The results of intestinal smooth muscle, kidney volume, intracystic and intracranial pressures are variable and so frequently negative as to be of no importance as an index of sensitization.

“8. A definite fall occurs in body temperature during the anaphylactic shock.”

Some of his other observations have been mentioned earlier in the chapter.

PROTRACTED ANAPHYLACTIC SHOCK IN THE RABBIT.—Coca (1927) gives an excellent description of this phenomenon. The animal may survive for varying lengths of time. Those that survive for days or weeks show marked loss of weight (cachexia).

LOCAL ANAPHYLAXIS OR ARTHUS PHENOMENON.—This has been extensively studied by Opie (1924). He concludes that it is an inflammatory reaction initiated by the meeting of antigen and antibody in the tissues. His studies include observations on reversed anaphylaxis. His published papers on anaphylaxis should be read by the student. Opie's work on the Arthus Phenomenon has been confirmed by Cannon and Marshall (1941).

Anaphylaxis in the Dog.—Acute anaphylactic shock was first observed in the dog by Richet and Portier (1902). Their description is given at the beginning of this chapter. The subject was investigated extensively by Biedl and Kraus (1909) and by Pearee and Eisenbrey (1910). They found that the characteristic physiological picture in dogs under ether anesthesia is a profound drop in blood pressure accompanied by extreme engorgement of the liver and splanchnics. They also reported a prolongation of the clotting time of the blood. A few other characteristic findings were observed. In 1913 Auer and Robinson observed heart block as occurring during acute shock. Manwaring et al. (1923, 1924)

and Simonds (1923, 1925) noted definite changes in permeability which Manwaring regards as characteristic of anaphylaxis in the dog.

In Manwaring's opinion, the explosive liberation of a toxin by the liver is responsible for canine anaphylactic shock. Simonds (1923) and Simonds and Brandes (1924) have offered a different explanation of the hepatic phenomena. They find that the walls of the hepatic veins contain a large amount of nonstriated muscle. They conclude from their experimental work that the drop in blood pressure and the engorgement of the liver observed in canine anaphylaxis result from increased pressure in the hepatic veins caused by the tetanic contraction of the smooth muscle present in the vessel walls.

Sherwood and Stoland reported that while animals injected with horse serum showed increased permeability changes, the phenomenon is observed in animals that have not become sensitized to horse serum; it can be demonstrated by perfusion with Locke's solution without antigen and is present in desensitized animals. The chronaxie studies of Stoland, Sherwood, and Woodbury (1931) indicate that following the injection of horse serum there develops an increase in irritability of the vagus which correlates with increased permeability of the tissues but does not always indicate that the animal is sensitive. They note also, that about 10 per cent of the sensitized dogs show no prolongation in the clotting time of the blood during shock. When the clotting time is prolonged, it is due, according to Stoland and Haughey (1932), to the liberation of a heparin-like substance by the liver. The rôle of heparin as a factor in blood coagulation has been studied extensively by Howell and Holt (1918) and more recently by Howell (1924).

Anaphylaxis in the Cat.—In 1900 Brodie called attention to the extreme sensitivity of normal cats to natural foreign proteins administered intravenously. Doses of foreign blood serum, egg white, etc., that are entirely nontoxic for guinea pigs, rabbits, dogs and other animals, caused a marked fall in arterial blood pressure in the normal anesthetized cat. For this reason, Manwaring (1910), Schultz (1911), Edmunds (1914), and later Drinker and Bronfenbrenner (1924) have encountered difficulty

in studying anaphylaxis in this animal. Kabler and Sherwood (1933) reinvestigated the subject after discovering that the Brodie reaction is not elicited by the intravenous injection of relatively large doses of crystalline egg albumen. They attempted to sensitize actively twenty adult cats and obtained entirely negative results. They were, however, able to sensitize *passively* 40 per cent of a series of cats to the same antigen by means of high titered immune rabbit serum. As a result of further work, they conclude that they were unable to sensitize cats actively because the latter did not produce sufficient antibodies. The results of their physiological studies of anaphylaxis in the passively sensitized cat under ether anesthesia are partly summarized as follows:

1. The characteristic blood pressure response is a profound drop followed by a slow return to normal. There may be a temporary return toward or to normal followed by a second drop and final return. This is spoken of as the "three-phase" curve.

2. During the period of low blood pressure there is a marked slowing of the heart rate.

3. There is a progressive decrease in the body temperature throughout the experiment.

4. There is an increase of intestinal pressure in the cannulated loop of small intestine. This suggests an active participation of the intestinal smooth muscle in the anaphylactic phenomenon.

5. The intraeystic (bladder) responses are variable.

6. The kidney volume is consistently decreased during shock. This suggests an active constriction of the renal blood vessels.

7. There is no characteristic prolongation of the clotting time of the blood such as is observed in the dog.

8. The smooth muscle reaction can be demonstrated *in vitro* when excised strips are removed from animals that can be shown to be sensitive by the intravenous injection of antigen.

Anaphylaxis in Rats.—Arthus (1903) was the first to study the effects of repeated injections of a foreign antigen into white rats. He claimed that he was able to produce specific sensitization. Novy and De Kruif (1917) were unable to confirm his results. In 1924 Parker and Parker, working in Zinsser's laboratory, succeeded in producing definite anaphylactic shock in white rats,

and demonstrated typical Dale reactions with the excised uterine horns. They state that anaphylactic phenomena observed in the white rat are similar to those described for the dog. Spain and Grove (1925) report that they were unable to sensitize guinea pigs passively with precipitating rat serum. Zinsser, Enders, and Fothergill (1939) suggest that this is due not only to the low antibody titer of the serum used, but, perhaps, also to the heterogeneity of the rat serum.

Anaphylaxis in Frogs.—In 1911 Friedberger and Mita inoculated a few frogs with 0.1 c.c. of sheep serum intravenously and after a period of seven to ten days gave a second injection of 0.25 to 0.5 c.c. of the same antigen. They report a definite decrease in pulse rate and note that the pulse is also weaker. Within two hours the animals were too weak to respond when stimulated mechanically. Kritevsky and Birger (1924) were unable to confirm these results. Goodner (1926), working in our laboratory, was likewise unable to confirm the clinical findings but he was able to confirm, by means of *in vitro* studies, the findings of Friedberger and Mita as to the sensitization of the frog heart. When specific antigen is added to a bath of Ringer's solution in which the excised heart from a sensitized frog is suspended, there "will occur an abrupt fall in amplitude and some decrease in rate" (Goodner, 1926, p. 338).

Anaphylaxis in Turtles.—The phenomenon of anaphylaxis in turtles was investigated by both Sherwood and Downs (1928) and Downs (1928). The results of the former deal with passive sensitization and may be summarized as follows:

1. Passive sensitization of turtles (ten out of a series of thirty-six) was accomplished by injecting them with high titered immune rooster serum.

2. When a shock dose of antigen was injected into the ventricles of the heart, a specific reaction occurred. It consisted of a marked slowing of the heart, increase in diastole, a decrease in amplitude, cardiac engorgement, and a sinking of the heart to a lower level. There was an apparent rise in tone probably due to the latter phenomenon.

3. In two of the turtles that showed positive results for passive sensitization, the hearts returned to normal and survived

eighteen hours with regular rhythm. They remained desensitized throughout this period.

4. Reversed anaphylaxis was not demonstrated in seven turtles, but a doubtful positive was obtained in one. The incubation period for the latter was two hours.

5. The shortest incubation period for passive sensitization was four hours, while the average was twenty-four to forty-eight hours.

6. High titered immune serum from *rabbits failed* to sensitize passively in a series of sixteen turtles.

The subject of active sensitization in turtles was investigated by Downs (1928). Her results may be summarized as follows:

1. Turtles may be actively sensitized to mammalian serum.
2. The heart in situ responds in a specific characteristic way to the injection of antigen used for sensitization.
3. Desensitization can be demonstrated.

4. Precipitins (titer 1:10 to 1:100) are present in the serum of a certain number of the actively sensitized animals, and this serum seems to confer passive anaphylaxis to normal turtles.

5. The specific response resembles vagus stimulation of the turtle heart.

Anaphylaxis in Chickens.—In 1926 Gahringer demonstrated clinical anaphylaxis in *chickens*. It is characterized by profound weakness, and lacrimation. The following year Hanzlik et al. (1927) described the smooth muscle reaction of the crop in pigeons. Sherwood (1928) reported upon his attempt to sensitize the embryonic chick passively. He found that high titered immune rooster serum will passively sensitize a very small percentage of 48- to 72-hour chick embryos. The characteristic response is a marked slowing of the heart with a final cessation in diastole. When the bath is changed, the heart will resume the normal rhythm. The latter is not affected by another dose of antigen. In other words, desensitization can be demonstrated. He produced also reverse anaphylaxis in a few embryos.

Anaphylaxis in Monkeys.—Monkeys are apparently very refractory to anaphylaxis. Coca (1927) and Zinsser, Enders, and Fothergill (1939) cite the work of Auer and others who were unable to demonstrate anaphylaxis in monkeys. Zinsser states that

in his studies one monkey developed, after prolonged sensitization, symptoms similar to those of serum sickness in man.

Kopeloff and Kopeloff (1936, 1939) seem to have produced both acute anaphylactic shock and the Arthus phenomenon in the Rhesus monkey. The functional and pathological changes observed in acute shock were prolonged clotting time of the blood, reduction in blood platelets and presence of skin hemorrhages. There were certain variable changes observed such as "emphysema, edema and hemorrhage of the lungs, edema and hemorrhages of the intestinal tract." Hemorrhages were also observed occasionally in other organs. The Schultz-Dale reaction was negative with both intestinal loops and uterine strips. They say there was no correlation found between the precipitin titer of the serum and the degree of sensitivity as determined by shock.

Anaphylaxis in Man.—Coca (1927) states that in his opinion "the existence of the condition of anaphylaxis in *human* individuals has not been demonstrated; in other words, no human pathological change has yet been shown to be the result of an anaphylactic antibody-antigen reaction." In his opinion anaphylaxis comparable to that observed in the lower animals has not been observed in man. He calls attention to extensive clinical observations that immediate symptoms of anaphylactic shock have not occurred as the result of a second injection of horse serum. He chooses to disregard the few cases that have shown symptoms of acute shock and does not classify serum disease, which occurs several days or weeks after a first injection of horse serum, as anaphylaxis. Zinsser, Enders, and Fothergill (1939) is of the opinion that the latter will ultimately be shown to be an expression of anaphylaxis in man.

Criteria of Anaphylaxis.—Wells (1929) calls attention to certain criteria which, if observed, will enable one to differentiate between true anaphylaxis and the phenomena resembling it which do not depend upon an antibody-antigen mechanism such as is described in the preceding pages. These criteria he lists as follows:

1. The observed toxicity of the injected material must depend upon the sensitization of the animal; i.e., the substance must not produce similar symptoms in non-sensitized animals.

"2. The symptoms produced must be those characteristic of anaphylactic intoxication as observed in the usual reactions with typical soluble proteins, being therefore the same for all antigens with the same test animal, but differing characteristically with each species of animal.

"3. It should be possible to demonstrate typical reactions in the nonstriated muscle tissue of the sensitized animal.

"4. The possibility that the observed symptoms are caused by capillary thrombosis or embolism must be excluded.

"5. After recovery from anaphylactic shock there should be exhibited a condition of specific desensitization to the same antigen under proper conditions.

"6. In addition to the above, it is usually, but not always, possible (a) to demonstrate passive sensitization with the serum of sensitized animals; and (b) to demonstrate amelioration or prevention of the bronchial spasm in guinea pigs by proper use of atropine and epinephrine."

Zinsser (1931) is of the opinion that criteria numbers 4 and 6b may be omitted, since he regards the others as adequate. He suggests that the criterion relative to passive sensitization should be modified since it has been shown that the species origin of passive sensitizing serum is important. He refers to observations previously cited that guinea pigs may be passively sensitized with antibacterial serum from rabbits, but not with antibacterial serum containing antibodies from a horse. It might also be added that Sherwood and Downs (1928) succeeded in passively sensitizing turtles to sheep and human blood with immune sera obtained from chickens, but were unsuccessful when they employed similar immune sera obtained from rabbits. Zinsser also calls attention to the newer work which has shown that clinical bacterial anaphylactic shock or the specific response of sensitized uterine horns can be demonstrated when the specific bacterial hapten (carbohydrate) is used. The whole antigen is, however, necessary for sensitization.

The Sensitizing Antibody.—It has been shown by Doerr and Russ (1909) that the sensitizing property of immune rabbit serum for guinea pigs parallels its precipitin content. Weil (1916) determined that the precipitate formed in the precipitin reaction could be washed free of serum and used to sensitize guinea pigs

passively. Those who hold to the plurality of antibodies for any one antigen have offered certain evidence against the assumption that precipitin and sensitizing antibody are the same. They point out that Longcope (1913), Spain and Grove (1925) and others were unable to sensitize guinea pigs with precipitating serum obtained from rats. This may be due to species differences as pointed out elsewhere in this chapter. Zinsser remarks that perhaps the low titer of precipitins in immune rat serum may be a factor. Furthermore, he does not regard certain quantitative discrepancies observed between the precipitating and sensitizing power of a serum, as significant since the formation of a visible precipitate depends not only upon the union of antigen and antibody but also upon many additional factors which have been discussed in preceding chapters. In fact one makes use of the failure of antigen-antibody mixtures to develop precipitates when he employs the "suppression phenomenon" of Landsteiner in the study of specificity. Opie (1924) in his study of the Arthus phenomenon noted definite correlation between the sensitizing and precipitating property of immune serum. According to Zinsser (1931), Ward and Enders carried out studies in his laboratory which indicate that the complement fixing titer of a serum is a more accurate measure of its sensitizing property than the precipitin titer. Since we accept as a working hypothesis for the present the unitarian concept of antibodies, it is obvious that we regard precipitin and anaphylactic antibody as the same.

Specificity of the Reaction.—The specificity of the reaction has been investigated extensively by Wells and also by Dale and others. They have found the reaction to be not only very specific but far more delicate than any biochemical tests known.

The Changes in Metabolism During Shock.—Wells (1929) in an excellent condensed discussion of anaphylaxis reviews the literature on the pathological, physiological and metabolic changes in anaphylactic shock. Major (1914) reports an increase in non-coagulable nitrogen, creatinine and urea in the blood; Abderhalden and Wertheimer note a decrease in the gas metabolism as a whole; Eggstein and others describe a marked acidosis associated with the asphyxia in guinea pigs, while McCullough and O'Neill report a marked increase in lactic acid. Among other

important papers cited by Wells are those of Hanzlik and De Eds (1926), who observed a marked change in endothelial permeability in anaphylactic shock, and Paterson and Levinson, who noted not only an augmented flow but an increased protein content of thoracic lymph during severe shock. More recently Kushnarjew (1930) calls attention to changes in the blood calcium and potassium during shock. We have suggested that since electrolytes enter into antigen-antibody reactions in general, the disturbance of electrolyte balance in or on the tissue cells may be an important factor.

The Nature of Anaphylaxis.—The evidence presented in the preceding pages, which seems to prove conclusively that the type of hypersensitiveness, discovered independently by Richet and Portier and by Theobald Smith, respectively, and named by the former anaphylaxis, is mediated by an antigen-antibody mechanism, may be summarized as follows:

1. Active sensitization can be produced by introducing into the tissues a true antigen only.

2. The incubation period which must elapse before specific hypersensitiveness develops corresponds to the required incubation period for the development by the body of specific antibodies and their fixation by the tissues.

3. Anaphylactic shock, as previously defined, is elicited by normally nontoxic doses of the specific antigen only, or, in the case of bacterial anaphylaxis, it may also be elicited by the specific bacterial polysaccharide used likewise in amounts nontoxic for the normal animal.

4. Blood from a sensitive animal is capable of passively sensitizing a normal animal only when it is obtained from the former at a time at which experience has indicated antibodies should be present. Where blood from sensitized rabbits is employed, it has been shown that its sensitizing power parallels its precipitin (antibody) content.

5. Weil showed that the washed precipitate obtained in the precipitin reaction can passively sensitize as well as actively sensitize a normal guinea pig. This suggests the identity of sensitizer and precipitin, since the precipitate is known to contain the latter bound to antigen.

SITE OF REACTION.—The second important point concerning the nature of anaphylaxis is that the site of the antigen-antibody reaction which leads to shock is in or on the tissue cells and not in the blood stream. This is supported by the following observations:

1. The introduction of sensitizing antibodies into the blood stream of a normal guinea pig does not render it immediately sensitive to antigen such as would be the case if the site of the reaction were in the blood.* Instead there must elapse four or more hours after the antibodies are introduced before the animal becomes sensitive to antigen. This is best explained by assuming that this time is required for the tissues to take up antibody.

2. Manwaring has shown that the blood of a sensitized dog may be replaced by the blood of a normal dog without impairing the sensitiveness of the former.

3. Doerr and others have shown that during passive sensitization, the animal becomes progressively more sensitive as the antibodies disappear from the blood.

4. Weil has shown that the injection of large amounts of antibody into a sensitive guinea pig interferes with the reaction. The reason is presumably that the circulating antibodies meet and react with the antigen before the latter can react with antibodies in the tissues.

5. Finally both Schultz and Dale have shown that segments of small intestine and also the uterine horns of sensitized guinea pigs perfused free of blood, removed from the body and properly suspended in Loeki's or Ringer's solution, will give a specific anaphylactic response when a nontoxic dose of antigen is added to the bath. Likewise Friedberger and Mita and also Goodner have shown that the excised sensitized frog heart gives a specific reaction when brought in contact with antigen.

It should be stated, however, that there are a number of investigators who have observed symptoms of anaphylaxis immediately after antigen and sensitizing serum were injected separately, but simultaneously, or mixed immediately before injection. Zinsser and also Wells do not regard these observations as invalidating the conclusion that in anaphylaxis the reaction occurs in or on the tissue cells. They suggest that the symptoms might be either anaphylactoid or toxic phenomena of undetermined cause.

*According to Bronfenbrenner immediate sensitization occurs if homologous instead of heterologous antiserum is used for passive sensitization.

While it is quite generally agreed that anaphylaxis is an antigen-antibody reaction occurring in or on the tissue cells, there is disagreement among immunologists as to what tissues are involved and how antigen and antibody cause the reaction. Pearce and Eisenbrey were unable to rule out the participation of intrinsic nervous mechanisms in canine anaphylaxis.

TISSUES INVOLVED IN ANAPHYLAXIS.—At the present time little is known as to how extensively the various tissues other than smooth muscle and, perhaps, the reticulo-endothelial system and the hepatic parenchyma are primarily involved in anaphylaxis. It is easy to recognize smooth muscle contraction but difficult to detect reactions or changes in other tissues and exceedingly difficult to be certain that any changes observed are primary and not secondary. Manwaring has long held that the hepatic parenchyma is primarily involved in anaphylaxis. Freund and others have shown that in immune animals antibodies are widely although unevenly distributed throughout the body, but, as to what cells other than those previously mentioned are involved and the kind as well as degree of relationship that exists between the various tissue cells and antibodies, nothing is known. Perhaps because there is considerable evidence that the reticulo-endothelial system is an important source of antibodies, there have been a number of attempts made to show that it is also an important primary site of anaphylaxis. Standenath (1923) and others report that blockade of the reticulo-endothelial system does not prevent shock. It is doubtful whether the observation of Kluge that he was able to prevent the Arthus phenomenon in sensitized rabbits by preliminary local injections of trypan blue into the area, can be regarded as proof of the primary participation of the reticulo-endothelial system in the Arthus phenomenon. It might be that the reaction between antigen and antibody in or on the clasmatoocytes, which are part of the reticulo-endothelial system present in the skin, leads to the liberation of toxic substances, which cause changes in the endothelium of the skin capillaries (which do not belong to the reticulo-endothelial system), but proof is apparently lacking. Since the endothelium of the ordinary lymph vessels, blood capillaries, arteries, veins and heart is not regarded as a part of the reticulo-endothelial system, the observation of changes in capillary permeability does not

prove that the reticulo-endothelial system is the primary site of anaphylactic shock. All that can be definitely stated is that Opie in his studies of the Arthus phenomenon showed that when antigen is injected into the skin of a sensitized rabbit there develops an inflammatory process. This, like all inflammation, is accompanied by local changes in vascular permeability along with other phenomena described in Chapter III.

Manwaring, Chilcote and Hosepian (1923) reported that when they perfused heart-lung preparations from sensitized dogs with Locke's solution containing antigen (horse serum), there was a 50 to 75 per cent reduction in the rate of perfusion flow, the lungs, which had been half inflated, failed to collapse when the tracheal clamp was removed, and a tracheal exudate appeared during the fourth minute which frequently amounted to 1,000 c.c. in seven minutes. These together with studies of the hepatic phenomena in dogs have led Manwaring to conclude that increased capillary permeability is the principal physiological change in anaphylaxis to which all other phenomena are secondary. It is interesting to note, however, that neither Manwaring nor Bally was able to demonstrate a similar pulmonary permeability change in the sensitized rabbit.

Furthermore, Sherwood and Stoland (1930, 1931) found that the permeability change, reported by Manwaring, Chilcote and Hosepian, develops as a result of the first injection of horse serum, can be demonstrated by perfusing with Locke's solution without as well as with antigen, is present before sensitization as judged by fall in blood pressure can be demonstrated, and is not affected by desensitization. Whether antigens other than horse serum will produce a similar change has not been determined. It would appear that the pulmonary permeability change described by Manwaring is at least not due entirely to an antigen-antibody-cellular reaction occurring at the time of the experiment, but results from the first injection of horse serum. Whether or not a similar fundamental change in permeability occurs in the canine liver as a result of sensitizing doses of horse serum is not known nor has it been investigated. It is conceivable, however, since horse serum has been used almost exclusively in the study of canine anaphylaxis that such a phenomenon may occur and be partly responsible for

the explosive edema of the liver that is observed in canine anaphylaxis. Since cells lining the liver sinusoids belong to the reticulo-endothelial system, it has been suggested that the explosive, hepatic edema points to these cells and the hepatic parenchyma as the primary site of anaphylaxis. On the other hand, Wells (1929) calls attention to evidence which indicates that the primary site of the antigen-antibody reaction is upon or within the cells of nonstriated muscle tissue. The evidence may be summarized as follows:

1. Smooth muscle removed from the body of sensitized guinea pigs will give specific anaphylactic contractions when nontoxic doses of the antigen used in sensitization are added to the bath. Desensitization can be demonstrated.

2. The inflation of the lungs of the guinea pig, which is the characteristic phenomenon of anaphylaxis in that animal, is due to the tetanic contraction of the smooth muscle of the secondary and tertiary bronchioles.

3. Simonds has shown that the walls of the hepatic veins of the dog are endowed with a large amount of smooth muscle. He offers experimental evidence which indicates that the characteristic drop in arterial blood pressure results from the increased pressure in the hepatic veins due to the tetanic contraction of the smooth muscle in their walls.

4. It has been established by Coca and others that the most important physiological anaphylactic phenomenon in the sensitized rabbit is a tetanic contraction of nonstriated muscle present in the walls of the pulmonary artery.

MECHANISMS OF THE REACTION.—While it is definitely established that anaphylactic shock is due to an antigen-antibody reaction occurring upon or within tissue cells of the body, the exact mechanism which elicits the physiological responses is unknown. At the present time there are two theories either of which appears to offer an adequate explanation of most of the observed phenomena. One theory assumes that the toxic stimulus results from physical (colloidal) changes, either at the surface or within the tissue cells, which result from antigen-antibody union. This explanation is either a part of or implied by the physical theory of Weil, the membrane hypothesis of Doerr, and the inflammation theory of Opie. A second theory formulated by Lewis and sup-

ported by certain experimental studies of Dale postulates the existence of an histamine-like substance (H-substance) in loose combination with the tissue cells. According to this theory the H-substance is released as a result of the antigen-antibody reaction. The symptoms of anaphylaxis are due to the H-substance thus liberated.

There are a number of observations which support the physical theory just mentioned. These may be summarized as follows:

1. It is definitely established by experiment that changes in the colloidal dispersion of both antigenic substance and antibody globulin accompany antigen-antibody reactions *in vitro*.

2. Specific agglutination and precipitation phenomena have been observed within animal tissues.

3. The results of Bronfenbrenner's (1914, 1915) investigations of the Abderhalden reaction suggest that the union of antigen and antibody is accompanied by physicochemical change which lowers the antitryptic index. This leads to the liberation of toxic products by the action of proteolytic enzymes.

4. Wells has called attention to experiments which show that slight changes in colloidal dispersion of plasma proteins may render them exceedingly toxic.

It would appear that such a theory might explain the smooth muscle reaction of Schultz and Dale as well as the clinical symptoms of shock.

One can also offer strong evidence in support of the histamine theory² of Lewis or some modification of his theory which does not restrict the toxic substance to histamine. The evidence which has been offered in support of Lewis' theory may be summarized as follows:

1. The symptoms of histamine shock in the guinea pig, rabbit, dog, and cat are very similar to those of anaphylactic shock in the respective animals.

2. Histamine will produce a contraction of the intestinal loop or uterine horn which gives a kymograph tracing identical in appearance with the specific anaphylactic response.

3. It is generally agreed that histamine and histamine-like substances are widely distributed throughout the tissues of the body.

Dale has shown that histamine is present in apparently loose combination with various fresh normal tissues (1939) and Drag-

stedt and Meade (1936) offer confirmatory evidence that the anaphylatoxin previously described by Dragstedt et al. is histamine.

Farmer (1939) reports that the injection of histamine into serum-sensitized guinea pigs renders the uterine horns less sensitive to antigen.

Sherwood, Stoland and Nelson (1941) have shown that the histamine reaction in turtles resembles the anaphylactic response and that turtles, unlike dogs, cats and rabbits, become refractory to histamine following recovery from the intracardiac injection of histamine. It is yet to be determined whether the refractory state is due to the liberation of histaminase.

Another report which points to the similarity between histamine and anaphylactic reactions is given by Andrus and Wilcox (1939). They report that the response of the coronary artery of hearts perfused with histamine was the same as the anaphylactic response.

In 1930 Best and McHenry demonstrated a histamine inactivating substance in horse lung and later in beef lung, liver and kidney. They noted that the inactivating substance was specific for histamine and have designated it *histaminase*. The early work on histaminase appeared to show that the inactivation of histamine occurred only *in vitro* but subsequent work by Karady and Browne (1939) indicates that inactivation occurs *in vivo*. They report that histaminase injected into guinea pigs fifteen minutes before the injection of a lethal dose of histamine prevented histamine shock in a majority of the animals. They also report that anaphylactic shock in sensitized guinea pigs is almost entirely prevented by an injection of histamine prior to the administration of the shock dose of antigen.

Since it would appear from the evidence submitted that either theory may be adopted to explain the anaphylactic response, it might be profitable to extend somewhat the discussion of each.

FURTHER DISCUSSION OF THE PHYSICAL THEORY.—

1. The physical theory assumes that physical changes occur either at the cell surface or within the cell and that such physical change supplies the toxic stimulus. It would seem that neither of these assumptions can be proved directly by experimental observation although they are in harmony with our present chemical and physiological concepts.

2. Desensitization is satisfactorily explained by assuming that the antibodies are either exhausted or neutralized during the reaction, hence, after recovery when a second dose of antigen is administered there are no antibodies available to react with antigen.

3. The specific tetanic contraction of smooth muscle does not explain the prolongation of clotting time frequently, although not invariably, observed in anaphylaxis. If one postulates physical changes occurring at the surface of or within the hepatic parenchyma cells or perhaps in the endothelium of the liver sinusoids, one might explain the appearance of a heparin-like substance which would cause the prolongation of the clotting time of the blood.

4. The inflammatory response observed in the Arthus phenomenon might be explained as due to similar changes in the skin. Not infrequently it is explained by assuming that the specific precipitate formed acts as a foreign body in causing inflammation. The exact mechanism involved is not understood.

FURTHER DISCUSSION OF THE HISTAMINE THEORY.—Certain objections to Lewis' theory that the reaction between antigen and antibody causes an explosive liberation of histamine which acts locally and perhaps always systemically have been summarized and discussed by Wells and others.

Among the objections are listed the failure of histamine to desensitize animals, its inability to produce coagulation changes of the blood similar to those observed in anaphylactic shock, and finally its ability to produce strong contractions in desensitized uterine horns. Wells calls attention to the fact that the first and third objections are not necessarily valid. Since the histamine is theoretically liberated as a result of an antigen-antibody reaction, the phenomenon of desensitization would mean an exhaustion of antibody and not an acquired tolerance for histamine. If antibody is exhausted during shock, then the second injection of antigen will find no antibody to unite with it and therefore no histamine will be liberated. A similar explanation is applicable to the third objection that histamine will produce a reaction in a desensitized uterine horn. If the antibodies present in the uterine horn are exhausted, then an antigen-antibody reaction cannot occur when antigen is added. Therefore no histamine will be liberated. The

capacity of the smooth muscle to respond to histamine need not be altered. In fact, one would expect the desensitized horn to react to histamine if it is added to the bath.

In regard to the second objection that histamine will not produce prolongation of the clotting time of the blood as is commonly observed in anaphylactic shock, this may be a real objection and again it may be found either that a toxic substance possessing properties of both histamine and peptone is liberated or that both chemical and physical mechanisms are involved. Rous and Gelding (1930) have offered experimental evidence which casts doubt upon the contention of Lewis that local vasodilatation after different tissue injuries is due to a single factor such as histamine. From their work they draw the following conclusions: "Experiment shows that the vascular contraction responsible for Bier's spotting prevails over the local vasodilatation caused by histamine pricked into the skin. The results raise doubts concerning the validity of the hypothesis referring all local vasodilatations to the action of a single chemical substance or set of substances (H-substance) liberated within the tissues."

The only serious physiological objections to regarding histamine as the toxic factor in anaphylactic shock have been raised as the result of studies carried out in our laboratory. These may be summarized as follows:

1. In histamine shock in the dog and rabbit each phase of the blood pressure response is decidedly shorter than in anaphylaxis. In the rabbit histamine frequently does not produce a drop in blood pressure below normal such as occurs almost invariably in anaphylactic shock. When histamine produces a lowering of the blood pressure in the rabbit, both the drop in pressure and the return to normal occur in much shorter time than in anaphylaxis.

In histamine shock in the cat the blood pressure tracing resembles that observed in anaphylaxis except that the phase of low pressure is longer (20 min.) in histamine shock than in anaphylactic shock (12.5 min.). It will be observed that this is the opposite of the findings in the dog and rabbit.

2. There is in histamine and anaphylactic shock a noticeable difference in the heart rate following the drop in blood pressure. In histamine shock in the rabbit 62.5 per cent showed an *increase*

in heart rate, whereas in anaphylactic shock 100 per cent of the rabbits showed a *decrease* in heart rate (Bally, 1929). There is also a difference in the degree to which the heart is slowed. Quite similar results were obtained by Kabler and Sherwood in their study of anaphylaxis in the cat. In the latter, when histamine slows the heart, the rate is reduced only moderately, while in anaphylactic shock the slowing is usually profound.

3. When one compares the vascular changes in the ear of the rabbit in histamine and anaphylactic shock, he observes definite differences in the intensity of the blanching reaction described by Bally. The injection of histamine, even though a fatal dose is given, never results in a complete obliteration of both capillaries and larger vessels since histamine is a capillary dilator. On the other hand, one notes complete constriction of both capillaries and larger vessels of the ear in rabbits during mild anaphylactic shock.

4. A comparison of kidney volume changes in the rabbit is also of interest. In the series of rabbits which Bally injected with histamine, none showed an increase, while 60 per cent of the injections resulted in a decrease of kidney volume. In a comparable series in which anaphylactic shock was studied, he reports that 39 per cent showed an increase and only 13 per cent a decrease in kidney volume. Similar differences in intracystic pressure in histamine and anaphylactic shock in the cat are reported by Kabler and Sherwood.

5. If human idiosyncrasies are mediated by the same mechanism as experimental anaphylaxis, then another reason presents itself for doubting the identity of histamine and the exciting agent causing shock. It is quite well established that when one injects a small amount of histamine into the human body an increase in gastric acidity results. This phenomenon is used in the diagnosis of pernicious anemia where an achylia is a characteristic finding. Since in asthma and hay fever the gastric acidity varies from zero to normal (Crip and Wechsler, 1931), it would appear that histamine is not liberated in the body in these allergic conditions.

6. Feinberg and Bernstein (1940) in a review of the literature on asthma and hay fever for 1939 conclude that more research is needed before the histamine mechanism of allergy can be accepted.

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CHAPTER XXVI

HYPERSENSITIVENESS DUE TO INFECTION

Allergy in Tuberculosis

Tuberculosis and Tuberculin Hypersensitiveness.—In 1891, Koch made the remarkable discovery that active tuberculosis in guinea pigs renders them extremely sensitive to extracts of old autolyzed glycerin broth cultures of the tubercle bacillus. He concluded that the active substance to which they become sensitive is a protein fraction of the bacterial cell and accordingly named it “tuberculin.”

TUBERCULIN OT, BE, AND TR.—In his original work, Koch employed a filtrate of a glycerin broth culture of the tubercle bacillus that had been incubated at 37° C. for six or eight weeks, sterilized and concentrated to one-tenth its volume. In an attempt to obtain a better yield of the specific active substance, Koch prepared a bacillary emulsion of the tubercle bacillus in glycerol and water and also a combined extract of finely pulverized bacteria. The former tuberculin he designated as BE and the latter as TR. It is interesting to note that of all Koch's preparations his original or old tuberculin, OT, is the one that has been most generally accepted. It is used quite extensively at the present time in the diagnosis and to a limited extent in the treatment of tuberculosis.

OBJECTIONS TO OT.—Certain objections to Koch's old tuberculin have been made by numerous investigators. It has been pointed out (1) that the meat broth employed as a culture medium contains protein derivatives and perhaps protein from the peptone and meat used in its preparation; (2) the old glycerin broth culture contains products of bacterial metabolism and bacterial autolysis other than the active tuberculin fraction.

LONG'S SYNTHETIC MEDIUM.—The first objection has been met by growing the tubercle bacillus in a synthetic medium. While a great many methods and formulae for preparing a medium of known chemical constitution have been used, the synthetic medium devised by Long is regarded in America as the first satisfactory one. It contains glycerol, asparagine, acid potassium

phosphate, ammonium citrate, sodium carbonate, sodium chloride, magnesium sulphate, and ferric ammonium citrate. The proportion and concentration of the constituents are such that the mixture is neutral and at the same time well buffered. Long states that growth in this medium yields 100 to 150 grams of moist tubercle bacilli per liter.

SYNTHETIC MEDIUM OF THE BUREAU OF ANIMAL INDUSTRY.—Another synthetic medium which Seibert (1934) says yields a heavier growth of tubercle bacilli than Long's medium has been developed by Dorset in the Bureau of Animal Industry. According to Seibert it differs from Long's medium in that it contains approximately two and one-half times more asparagine and twice as much glycerol as the latter. It contains also 10 per cent dextrose but no carbonate or ammonium ion except that which might be derived from the asparagine. This is the medium which Seibert (1934) and Long (1934) recommend for the production of a standard tuberculin which is now called PPD (Purified Protein Derivative of tuberculin).

The second objection has been overcome by Long and Seibert (1926) and Seibert (1932, 1934), although Long states that the greater part of the chemical work was carried out by Seibert. They grew the tubercle bacillus in Long's protein-free synthetic medium and studied the chemical changes that occurred. From these and other studies the following important results were obtained:

THE ACTIVE SUBSTANCE, ITS RECOVERY AND PROPERTIES.—1. That the tuberculin activity of filtrates develops simultaneously with the appearance of protein in the medium. The protein is derived apparently by autolysis or extraction of the tubercle bacillus.

2. If the tuberculin is subjected to dialysis, there remains in the dialyzing sac only the active substance together with protein and the polysaccharide peculiar to the tubercle bacillus.

3. The protein and active substance are completely precipitated out of the filtrate by full saturation with ammonium sulphate or by trichloroacetic acid.

4. The ammonium sulphate precipitate contains both coagulable and noncoagulable protein and in addition protease. The activity is destroyed by pepsin which splits the protein, but the activity

is not affected when the protease is destroyed by trypsin or erepsin. This indicates that the active substance is a protein.

5. The active substance, in the presence of N/10 hydrochloric acid, is not destroyed when heated under fourteen pounds pressure at a temperature of 120° C. for four hours. It is hydrolyzed by acids having a strength of N/6 or above.

6. Seibert attempted crystallization of the protein and succeeded in inducing a portion of the active substance to crystallize in needle form. This active crystalline substance possesses the property of an albumin.

7. They observed that a greater yield of active substance is obtained in filtrates of the old synthetic broth culture than from extraction of tubercle bacilli. This probably explains why tuberculin OT is generally preferred to BE or TR.

8. More recently Seibert and Munday have combined ultrafiltration with trichloroacetic acid precipitation in the preparation of a purified active protein fraction.

9. Masucci and McAlpine (1930) made use of the suggestions of Seibert in preparing a tuberculin which Funk and Huntoon (1930) have designated as MA-100 in accordance with the Johnson chart for the chemical analysis of the tubercle bacillus.

TPT. OF SEIBERT AND MUNDAY.—The new tuberculin protein prepared by Seibert and Munday (1932) is designated by them as TPT. These letters indicate that the product is tuberculin protein precipitated by trichloroacetic acid. In their opinion it is superior in some respects to TPA which they prepared in 1931 by precipitating old tuberculin with ammonium sulphate. Seibert and Munday describe their method of preparing TPT as follows: "Virulent tubercle bacilli (Saranac Lake Strain H37 has been used in our work) are grown upon Long's synthetic medium for eight weeks at 37.5° C. (more protein is obtained by allowing the culture to grow for eight to twelve weeks instead of the usual four to six weeks). The bacilli are removed by filtration through China silk or a Buchner funnel and then through a Mandler filter. The clear yellow tuberculin, preserved with 0.5 per cent phenol, is then concentrated by ultrafiltration on a 12.5 per cent gun-cotton-glacial-acetic-acid membrane, and washed with 0.5 per cent phenol solution by continued ultrafiltration until the filtrate is chloride- and iron-free, and then filtered. (It has not yet been

determined whether for routine preparations this additional washing process is absolutely essential before precipitation. In order to obtain the quantitative results given in this paper, it was, of course, necessary.) The protein is then precipitated from the pure colloidal solution in a final concentration of 10 per cent trichloroacetic acid until the wash is clear and colorless. The drained precipitate is partly dried in vacuo and then ground to a fine powder under larger volumes of ether, which simultaneously dehydrates the product and removes the trichloroacetic acid. A fine cream-colored powder, TPT, is thus quickly obtained."

The product is rendered completely soluble in water by cautiously adding a few drops of N/10 alkali and immediately neutralizing it with HCl. They prepare stock solutions in 0.9 per cent saline containing 0.5 per cent phenol.

The underlying principles of the above technique may be summarized as follows:

1. Using a standard virulent culture of the tubercle bacillus and a synthetic medium, a tuberculin is produced.

2. The bacteria are removed by filtration.

3. By means of ultrafiltration the resulting tuberculin is concentrated and at the same time rendered free of all substances except the active colloidal tuberculin protein and the ever present polysaccharide found in old tuberculin.

4. The active tuberculin protein is separated from the polysaccharide by precipitation with trichloroacetic acid.

5. The precipitate is washed, dried, and dissolved in saline to which phenol is added as a preservative.

The above treatment does not denature or in any way injure the tuberculin protein.

SEIBERT'S SOT AND SOTT OR PPD.—Seibert prepared also an old tuberculin (SOT) from cultures grown in a synthetic medium. She next concentrated and purified SOT by ultrafiltration and precipitated the active principle with trichloroacetic acid. The precipitate was treated with ether to remove the acid and to dehydrate the protein. This purified protein derivative of the tubercle bacillus she calls SOTT (synthetic medium old tuberculin precipitated with trichloroacetic acid). In 1932 she prepared SOTT from cultures grown on Long's medium, but later she has adopted the synthetic medium used by the Bureau of Animal Industry.

This product is now called Purified Protein Derivative or PPD. The method of its manufacture is described in an excellent paper by Reichell and Clark (1934).

MA-100 OF FUNK AND HUNTOON.—To prepare MA-100, a culture is grown in Long's medium, rendered bacteria-free by filtration through a Berkefeld filter, and the active tuberculin protein precipitated from the medium at a given isoelectric point by ammonium sulphate. The precipitate is purified by eight successive precipitations. The precipitate obtained in the operation is dissolved in distilled water and the protein precipitated by ethyl alcohol. Subsequent purification of the protein is accomplished by a series of isoelectric precipitations.

Mariette and Fenger working in collaboration with Funk, Huntoon and White (1932) have carried out extensive studies of MA-100 proteins obtained from the human, bovine and avian tubercle bacillus and from a culture of the timothy bacillus. In preparing the purified tubercle bacillus protein they apparently employed a modification of Masucci's and McAlpine's method, since they say that precipitation at a given isoelectric point was accomplished by means of glacial acetic acid. They recommend the use of MA-100 tubercle bacillus (human) protein in diagnostic work. The results of their studies and also those of Funk and Huntoon (1930) are discussed later in this chapter.

RELATIONSHIP OF MOLECULAR WEIGHT TO SENSITIZING PROPERTY.—In 1933 Seibert reported upon the relationship between the sensitizing properties of tuberculin and its molecular weight. Numerous studies had indicated that while OT, TPA, TPT, and SOTT or PPD are all capable of eliciting specific allergic reactions in tuberculous animals, they differ markedly in the degree of sensitization to tuberculin produced by repeated injections of the respective products into the animal body. Since Svedberg had emphasized the fact that in dealing with purified proteins the final molecular size of the latter will depend upon the method of isolation, it occurred to Seibert that differences in the molecular weight of OT, TPA and TPT might result from the methods used in their preparation and that some correlation between the molecular weight and sensitizing property might exist. Her experimental investigation led her to believe until recently (1941) that such is the case.

These earlier results of Seibert have been reinvestigated by Seibert, Pedersen and Tiselius (1938) by means of the ultracentrifuge and electrophoresis. They have obtained from TPA an antigenic protein with a molecular weight of 32,000 and from PPD a protein with a molecular weight of 17,000 to 18,000. These two proteins would elicit both local and systemic reactions in tuberculous guinea pigs. Positive skin reactions were obtained with substances of smaller molecular weight obtained from old tuberculin.

Seibert (1941) has since modified the method of PPD production by carrying out the procedure at 4° to 5° C., using less heat and weaker acid solution and drying the final product by the lyophile process. The product obtained by this procedure is twice as potent biologically and much purer than previous preparations. The surprising thing is that while the molecular weight is only about 10,500 this more potent tuberculin is more antigenic than the previous PPD protein preparations, having a molecular weight of 17,000 to 18,000. This has led Seibert to think that the size of the molecule is not so important in antigenicity as she previously thought. She is now of the opinion that the potency is inherent in one part of the protein molecule while the antigenicity is dependent upon another part of the same molecule.

The advantage of the purified protein derivative of tuberculin (PPD) lies in the fact that it can be injected repeatedly into the same person without causing skin allergy to develop. The method of preparation of the new PPD is given by Seibert and Glenn (1941). Just why some of these highly purified fractions give a higher percentage of reactors in the second injection than previous preparations is unknown. Seibert (1941) says that this point is being investigated.

CHEMICAL NATURE OF PPD.—Seibert (1941) says that the new, dried PPD is almost colorless, quite soluble in water, and contains approximately 1.2 per cent nucleic acid and 5.9 per cent polysaccharide. Its potency is twice that of the previous PPD. This is indicated by the fact that 0.00001 mg. produces as strong reactions in sensitive patients as 0.00002 mg. of the previous product.

According to Chase and Landsteiner (1939) the separation of tuberculin into two fractions, one responsible for skin reactions and the other for systemic reactions, is reported by Maschmann

(1937). The skin reacting fraction is broken down by trypsin or papain while the other fraction is not acted upon by proteolytic enzymes.

Classification of Standardization Methods.—An excellent discussion of present methods used in the standardization of tuberculin is included by Long* (1925) in his report of a new method of assaying the strength of tuberculin by means of the "spermatocyte reaction" in tuberculous guinea pigs. Long classifies the methods in use at the present time as follows:

"I. Hypersensitiveness of the Tuberculous Guinea Pig.

1. The lethal dose. Method of:

Koch

The Institute for Experimental Therapy, Frankfurt
a. M.

The United States Bureau of Animal Industry.

2. The skin test.

Method of Lewis and Aronson.

"II. Antigenic Capacity of Tuberculin in Serum Reactions.

1. The precipitin test.

Method of Dreyer and Vollum.

2. The complement-fixation test.

Method of Watson and Heath."

The following description of these methods is based upon Long's report.

KOCH'S METHOD.—The discoverer of tuberculin according to Long, "considered a preparation of tuberculin satisfactory if 0.5 c.c. or less would kill in six to thirty hours, with characteristic pathologic change, a guinea pig infected one month previously with tuberculosis."

METHOD USED IN THE INSTITUTE OF EXPERIMENTAL THERAPY, FRANKFURT.—This is a modification of Koch's method suggested by Doenitz (1921). A large number of guinea pigs, fifty or more, are infected with tubercle bacilli. After definite symptoms of the disease develop two series of six guinea pigs each are selected. One series is injected subcutaneously with varying amounts of Koch's OT while the second series is injected with corresponding amounts of the tuberculin to be standardized.

*Long: J. Infect. Dis. 37: 368, 1925.

The smallest dose of each that kills within twenty-four hours is determined. The minimum lethal dose of Koch's OT is the standard with which the M.L.D. of the new product is compared. If the latter is larger than the former, then the new product is below standard, while if it is smaller it must be diluted to correspond to the strength of the old tuberculin used as a standard.

METHOD OF U. S. BUREAU OF ANIMAL INDUSTRY.—Apparently this is also a modification of Koch's method suggested by Schroeder and Brett (1919). Tuberculous tissue from guinea pigs is inoculated into a series of normal guinea pigs. As soon as these latter become sensitive to the extent that 0.25 gram of OT per 500 grams body weight will kill 4 out of 6 pigs within twenty-four hours, the remaining pigs are ready for use in standardizing a new product. They are divided into groups of six. Each animal of group I is inoculated intraperitoneally with 0.25 gram of the standard OT per 500 grams body weight and each of the other groups is inoculated with a corresponding amount of the tuberculin to be tested. Long says that "a preparation of tuberculin is considered as passing the test if under the conditions of the experiment it kills at least one-half as many guinea pigs as the standard tuberculin, with the characteristic lesions of tuberculin death."

SKIN TEST METHOD OF LEWIS AND ARONSON.—In this method tuberculin hypersensitiveness is produced in guinea pigs by inoculating intraperitoneally a series of animals, ranging in weight from 200 to 300 grams each, with 0.1 mg. of virulent tubercle bacilli. After three weeks they are ready for use in the standardization of tuberculin. They are divided into groups of six and decreasing amounts of a standard tuberculin are injected *intracutaneously* after the method of Roemer. Each of the other series is injected in a like manner with corresponding amounts of tuberculin of unknown potency. In the series cited by Long the amounts used for *intracutaneous* injections were 0.02, 0.01, 0.005, 0.002 and 0.001 mg., brought up to a total volume of 0.1 c.c. by the addition of a diluting fluid. The results were read after forty-eight hours and recorded as 1, 2, 3, or 4, the last named figure (4) indicating the strongest reaction.

STANDARDIZATION OF TUBERCULIN BY THE PRECIPITATION METHOD OF DREYER AND VOLLUM.—Immune serum for use in the test is prepared by injecting defatted tubercle bacilli in amounts varying

from 0.1 mg. to 225 mg. into a horse. A period of three months is employed for immunization. When the precipitin titer is sufficiently high, the horse is bled and the serum obtained for use in the test. The authors find it necessary to employ the optimum proportion technique of Dean which is discussed in the chapter on Precipitins. They choose the Frankfurt standard tuberculin as the standard for comparison, and arbitrarily assign to it a value of 100 units per cubic centimeter. "The value of any other tuberculin is inversely proportional to the smallest amount required to produce the same degree of precipitation as the standard with the same quantity of serum under the same conditions." In the opinion of Dreyer and Vollum the strength of a tuberculin determined by the precipitin method compares favorably with the strength determined by skin tests on human subjects.

COMPLEMENT FIXATION METHOD OF WATSON AND HEATH.—Standardization of Tuberculin by Complement Fixation: This method is credited to Watson and Heath. They prepare an antiserum by injecting intravenously into a horse 1 mg. of virulent, moist tubercle bacilli followed in fourteen days and twenty-one days by 2 mg. and 3 mg., respectively, of the same kind of material. The concentration of complement fixing antibody reaches a peak during the seventh week. At this time the titer is said to be about 0.0066 or 150 units per c.c. when titrated against a fixed dose of standard tuberculin. They state that a fixed dose of 20 units of antibody is the most suitable one for titration of tuberculin. In standardizing commercial tuberculins they determine the minimum amount of the tuberculin which, when mixed with 20 units of antiserum, will completely fix the unit of complement used. This amount of tuberculin is said to represent one active unit.

OBJECTIONS TO LETHAL TESTS.—*Criticism of tests based upon lethal doses of tuberculin.* As regards the three methods, which it will be recalled involve the death of the guinea pig, there are too many unknown factors that affect the outcome of the test. Among these is the extent of the tuberculosis within the guinea pig. This cannot be known or controlled. In Long's opinion "individual variation (in animals) is so great as to rob the test of much of its value."

The result of Koch's test does not establish a unit: it is quantitative only in a "pass or fail" manner. Long states that the test will not differentiate between two preparations of tuberculin, one of which might be twice as potent as the other. While the Frankfurt method attempts to avoid this criticism, it does so only by introducing an extremely "laborious process of comparison with a standard tuberculin on a large series of animals." Although the test employed by the United States Bureau of Animal Industry is superior in certain respects to other "lethal dose tests," it too requires a large number of animals and is also quite laborious. Long calls attention to the fact that while a test of this type establishes a "standard which must be met before a tuberculin is acceptable, it does not establish a unit which can be used in measuring doses."

ADVANTAGES AND DISADVANTAGES OF THE VARIOUS TESTS.—

Criticism of the intracutaneous test.

1. The technique is simple and economical of both time and animals.
2. It would seem more logical to standardize in respect to the skin allergy of a test animal since the product is to be used to determine the skin allergy of patients.
3. Long and others feel that one serious objection to the test is that in a series of tuberculous guinea pigs the reactive capacity of the skin varies tremendously. On the other hand, Seibert and Munday (1932) find that when one pound guinea pigs are inoculated subcutaneously with 0.1 mg. of strain H37, they develop quite uniform skin sensitivity. In regard to the standardization of tuberculin they say "since it is possible to obtain consistent results in sensitive tuberculous guinea pigs by means of the intracutaneous test, and since the chief use of tuberculin at present consists in diagnostic skin reactions, it is advisable to base the standardization, so far as possible, upon the intracutaneous test as has been maintained by Aronson (1926), Okell and Parish (1927) and Funk and Huntoon (1930)."

Criticisms of the complement fixation and precipitin methods.

The chief objection to both of these methods of standardizing tuberculin is that neither complement fixing nor precipitating antibodies are known to be involved in the tuberculin reaction.

In fact, Parker and others working in Zinsser's laboratory have shown that so far as the precipitin reaction is concerned, it is dependent upon an entirely different substance in the tuberculin from that which elicits a positive skin reaction in tuberculous animals. Furthermore, both complement fixing and precipitating antibodies can be produced by injecting tuberculin into normal animals, but such treatment does not produce tuberculin hypersensitiveness such as is observed associated with active tuberculosis.

STANDARDIZATION BY LONG'S SPERMATOCYTE REACTION.—In this test 10 animals are required. Eight are infected by injecting one-half milligram of the Saranac laboratory strain R_1 into the groin. This leads to the development of a caseous nodule in the regional lymph nodes within one month, but the process does not become generalized. The animals are injected in duplicate with 0.01, 0.001, 0.0001 and 0.00001 mg., respectively, with the tuberculin to be tested. The two normal controls are inoculated with the strongest dose. The volume of each dose is made up to 0.1 c.c. Injections are made into the middle of one testicle. The reaction may be read after thirty-six hours or in from two to four weeks, the animals being killed and paraffin or celloidin sections prepared.

SPERMATOCYTE TUBERCULIN UNIT.—The spermatocyte tuberculin unit according to Long "is that quantity of tuberculin just sufficient to abolish spermatogenesis in the majority of tubules on injections of a volume of 0.1 c.c. into the testicle of a 400 gram guinea pig with a mild localized tuberculosis of one month's duration. Microscopic section of the testicle (middle cross-section) one month after the injection of one unit or more shows the majority of the tubules atrophic and lined only by spermatogonia. No normal spermatocytes, spermatids, or spermatozoa are present. The test is controlled by examination of the noninjected opposite testicle and of the injected testicle of a noninfected animal, both of which should show normal spermatogenesis."

In Long's opinion this unit is useful in standardizing tuberculins for use in either therapeutic or diagnostic procedures. He states that tuberculins such as OT and BE contain 10,000 and 1,000 spermatocyte units, respectively, per cubic centimeter.

DEPENDABLE METHODS OF TUBERCULIN PRODUCTION AND STANDARDIZATION.—It would appear from the research so far reported in this chapter that there are at present four methods available for the production of fairly pure tuberculin. The products of these methods have been designated as MA-100, TPA, TPT, and SOTT or PPD, respectively. A new method of producing PPD has been suggested by Seibert and Glenn (1941). It is discussed briefly later in this chapter. Because all of these tuberculins except the purified protein derivative are more or less antigenic and lead to the development of skin hypersensitiveness in some individuals when injected repeatedly, Long (1934) recommends that the latter (PPD) be used in determining the presence of tuberculin allergy in man.

There have been developed also two methods of standardization that give what appear to be consistent and dependable results. These may be designated as the standardized intracutaneous method of Aronson (recommended by Seibert) and the spermatoocyte reaction of Long.

Diagnostic Tuberculin Tests.—In diagnostic work both local and systemic reactions have been used to determine tuberculin hypersensitiveness. Among the tests based upon local allergic phenomena may be mentioned the cutaneous tuberculin test of von Pirquet, the percutaneous tuberculin reaction of Moro, the intracutaneous test of Mantoux and the ophthalmo-tuberculin reaction of Calmette or a similar one called the conjunctival-tuberculin reaction of Wolff-Eisner. Koch first employed a subcutaneous tuberculin test in which a positive reaction is indicated by both a local and a systemic reaction. The technique and allergic phenomena involved in each of the various tests may be summarized as follows:

THE VON PIRQUET TEST.—The technique of the von Pirquet test consists in first washing the inner surface of the forearm with ether and applying two drops of Koch's OT to the surface of the skin about 10 cm. apart. Then with a sharp instrument one scarifies by pressure and rotation a small area about midway between the two drops of tuberculin. This serves as a control. Similar scarifications are then made through each drop of tuberculin after which the latter is allowed to dry. While dressing is

not necessary, the areas are frequently covered with small pieces of sterile gauze held in place by adhesive tape.

The result of the test according to von Pirquet may be a traumatic, a negative or a positive reaction. The traumatic reaction may appear as an area of hyperemia or the latter may surround a small wheal. These disappear usually within twenty-four hours. There may remain a slight redness and a small crust for a few days. The negative reaction may also resemble the traumatic reaction. According to von Pirquet the redness and swelling should not exceed 5 mm. after twenty-four hours and this should not persist.

Positive reaction. This begins as a rule after a latent period of several hours. It is characterized by an area of edema (swelling) one or more centimeters in diameter accompanied as a rule by hyperemia (redness). The swelling reaches its height in about forty-eight hours and then begins to subside. The color may become darker and even develop a yellowish tinge. The swelling usually disappears within a few days to one week and there remains not infrequently an area of pigmentation.

MORO'S PERCUTANEOUS TEST.—To use the percutaneous test of Moro one must prepare a salve by triturating equal parts of old tuberculin and lanolin. A piece of this about the size of a pea is rubbed for one minute into an area of skin about 5 cm. in diameter over either the pectoral region or the epigastrium. The salve is then allowed to dry for about ten minutes. A positive reaction is indicated by the development of nodules, a rash or of small vesicles in the area tested. The test is not in use at the present time.

WOLFF-EISNER TEST.—The conjunctival-tuberculin test of Wolff-Eisner is made by instilling one drop of a 1 per cent solution of OT into the conjunctival sac. When positive, the reaction appears as either a simple hyperemia, an enlargement of the follicles or a severe conjunctivitis coming on within sixteen to twenty-four hours. It is no longer regarded as safe for use in testing children because of severe reactions and permanent injury.

PATCH TEST.—Pearse, Fried, and Glover (1940) state that the Volmer patch test is as reliable as the Mantoux test.

MANTOUX TEST.—The intracutaneous test of Mantoux is now regarded by many as the most satisfactory method of determining tuberculin hypersensitiveness. The dosage employed by different

individuals and the results which they report may be summarized as follows:

Funk and Huntoon's Results With OT and MA-100.—In 1930 Funk and Huntoon* performed intracutaneous tests in 441 individuals using both Koch's old tuberculin and the new product MA-100. In regard to the appearance of the reaction they say, "The reaction is usually an erythema surrounding an area of edema, although occasionally the edema is larger in area than the erythema, or rarely, edema without erythema. The erythema may vary from a very faint blush to a dark red, may be diffuse in character, or very sharply limited. The edema varies from a barely perceptible (to the touch) thickening to a marked induration several millimeters in height." The reactions come on within twenty-four hours; about half of them begin to fade before, and all after, forty-eight hours. They state that pigmentation occurs but is less likely to follow with the protein than when OT is used.

DOSAGE EMPLOYED.—They adopted a dosage of 0.00001 c.c. of OT and 0.0005 mg. for the MA-100. These respective tuberculins were diluted so that 0.05 c.c. contained the test dose for the Mantoux test. They used tuberculin syringes (1 c.c.) of the Luer type with a number 24-gauge $\frac{3}{4}$ inch needle. The level of the needle was turned up, i.e., in line with the graduations. After washing the anterior surface of the forearm with alcohol and drying it, they inserted the needle into the skin until the bevel was completely covered although dimly visible. The test dose contained in 0.05 c.c. of solution was then injected, producing a small wheal about 5 to 7 mm. in diameter. The arm was then wiped with alcohol.

READING THE REACTIONS.—They made the first reading between twenty-three and twenty-five hours after the injection and a second reading after forty-eight hours. If the first reading was negative they made a second test injection using 0.1 c.c. of the same dilution. In other words, they doubled the dose for the second test. In reading the reactions they noted the intensity of the color of the hyperemia and measured the longest and shortest diameters of both the erythema and edema. The latter information was recorded in millimeters.

The measurements of the reactions observed in 218 individuals positive to both OT and MA-100 showed an average diameter of

*Funk and Huntoon: J. Immunol. 19: 237, 1930.

17 mm. for the erythema and 13.4 mm. for the edema produced by OT as compared with 20.1 mm. for the erythema and 11.2 mm. for the edema caused by the MA-100. They observed no necrosis in any of the reactions with MA-100 although the induration persisted for several weeks in a few individuals. Occasionally superficial necrosis was observed in the reactions produced by OT. No general symptoms were noted in any of the individuals tested.

INCIDENCE OF REACTIONS IN KNOWN AND SUSPECTED CASES OF TUBERCULOSIS.—In a series of 204 cases of pulmonary tuberculosis 94.1 per cent were positive to MA-100 while 90.6 per cent were positive to tuberculin OT. In 57 cases of suspected tuberculosis the percentage of positive reactions was 92.9 with MA-100 and 91.2 with tuberculin OT.

INCIDENCE OF REACTIONS IN CLINICALLY NONTUBERCULOUS CASES.—It is interesting to note that in a series of 137 adult patients, all clinically *nontuberculous*, Funk and Huntoon found 81 per cent positive reactions to MA-100 and 71.5 per cent to tuberculin OT. In another series of 25 cases also clinically *nontuberculous* the percentages of positives were 80 and 68, respectively. They also tested 43 children whose ages varied from one to twelve years (average 5.3 years) and found 25.5 per cent positive to MA-100 and 16.2 per cent to tuberculin OT.

NONCORRELATIVE REACTIONS.—In the series of clinically tuberculous cases cited above there were 12 individuals who reacted to tuberculin MA-100 and not to OT and 5 who reacted to the latter but not the former.

CASES OF TUBERCULOSIS THAT DID NOT REACT.—There were also 13 clinically tuberculous individuals who did not react to either MA-100 or OT. Eight of these patients were quite ill, three of them were described as *cacheectic*. The condition of the five remaining individuals was regarded as either fair or good. In this connection Rich and McCordock (1929) as well as others call attention to the fact that tuberculin tests may be negative in 3 to 5 per cent of the cases of *miliary tuberculosis* and may be suppressed in certain of the *exanthematous diseases* as well as during labor and the *puerperium*.

Mariette and Fenger's Results With MA-100 and OT.—More recently Funk, Huntoon and White have collaborated with Mariette and Fenger (1932) in an extensive study of the skin reactions

to MA-100 and OT in tuberculous and nontuberculous subjects. In this work the skin reactions with OT and the MA-100 proteins of the timothy bacillus and the human, bovine and avian tubercle bacillus were studied in 225 individuals by Funk, Huntoon and White and in 3,454 by Mariette and Fenger. They employed the following dosage in these investigations:

Tuberculin OT "Dorset": Dose 0.05 c.c. = 0.00001 c.c. commonly designated at 0.01 mg.
 MA-100: Human tubercle bacillus protein: 0.05 c.c. = 0.0005 mg.
 MA-100: Bovine tubercle bacillus protein: 0.05 c.c. = 0.0001 mg.
 MA-100: Avian tubercle bacillus protein: 0.05 c.c. = 0.001 mg.
 MA-100: Timothy bacillus protein: 0.05 c.c. = 0.01 mg.
 If the reaction is negative at the end of forty-eight hours, repeat with double the dose.

The statistics of Funk, Huntoon and White are fairly representative of the general results and may be tabulated as follows:

TEST MATERIAL	CLINICALLY TUBERCULOUS	CLINICALLY NONTUBERCULOUS
	PER CENT POSITIVE	PER CENT POSITIVE
Tuberculin OT "Dorset"	87.4	68.5
MA-100 Human tubercle protein	84.6	73.1
MA-100 Bovine tubercle protein	82.0	69.5
MA-100 Avian	94.0	87.0
MA-100 Timothy protein	95.7	87.9

In a larger series studied by Mariette and Fenger, the MA-100 human tubercle protein gave about 10 per cent more positives among adults and 2 per cent among children classified as sanatorium patients than tuberculin OT. In the general population Mariette and Fenger report that tuberculin OT gave 27.2 per cent positives among adults and 6.9 per cent positives among children, whereas MA-100 human tubercle protein yielded 39.9 and 8.5 per cent positives, respectively, in the same individuals. From these studies they draw the following conclusions:

1. That MA-100 human tubercle protein is probably more sensitive than OT.
2. The initial and repeat doses may be administered safely to tuberculous patients.
3. Larger doses are not necessary to detect the majority of tuberculous individuals.
4. Large doses of MA-100 proteins give nonspecific results.
5. There is a protein common to all members of the acid-fast group which when administered in large doses gives a reaction similar to a positive tuberculin (OT) reaction.
6. They regard MA-100 tubercle protein as purer and better than OT for determining tuberculin hypersensitiveness.

Slater and Jordan's Studies of the von Pirquet and Mantoux Tests.—Slater and Jordan (1932) made a comparative study of the von Pirquet and Mantoux tuberculin tests in school children. For the former they employed Koch's old tuberculin and the usual von Pirquet technique. In conducting the Mantoux test they injected intracutaneously 0.1 c.c. of a 1:1,000 dilution or approximately 0.1 mg. of Koch's old tuberculin and read the results forty-eight hours later. Both tests were given at the same time, one on each forearm. In all they tested 1,006 white children of whom 134 reacted to one or both tests. It is of interest to note that a physical examination and x-ray plates of the chest of each of the 134 positive reactors revealed lesions of tuberculosis in only fifteen individuals. In regard to the relative delicacy of the two tests they report that of the 134 giving positive tests 42 reacted to the Mantoux but not to the von Pirquet, 63 reacted to both, while 29 were positive to the von Pirquet but not to the Mantoux. These children were all from schools in a district where during the last five years the death rate from tuberculosis has been less than 25 per 100,000 population. In contrast to this they tested 62 children from 19 families in which known cases of open tuberculosis were present. Fifty-one of the children reacted to one or both tests. Of these positive reactions six were to the Mantoux but not to the von Pirquet and two to the von Pirquet, but not to the Mantoux. In the author's opinion both tests are good, but not infallible, the Mantoux being more sensitive and perhaps better adapted for older children, while the von Pirquet gave good results with younger children.

Survey to Determine Primary Tuberculosis Infection Attack Rate.—Stewart, Harrington, Myers, Boynton, and Streukens (1939) employed 0.1 mg. of old tuberculin intradermally to test 3,868 persons, representing five different groups. They estimate that the children tested became infected with tubercle bacilli at the rate of 0.8 per cent of the group annually. The parents had an attack rate of about 1.6 per cent annually. Among college students they report that 5.8 per cent became sensitive to tuberculin during their four-year course at the university. The most disturbing figures they give pertain to medical students. They state that of 265 who gave negative reactions to 0.1 mg. of old tuberculin at the beginning of their senior year, 118 were positive at the end

of that school year. This constituted an attack rate of 44.5 per cent which was sixteen times the annual rate noted for the first three years of the course. They say that this rate of 44.5 per cent is fifty-six times the infection attack rate they found for children in the city schools who resided in private homes. Stewart and his associates also report high attack rates in private hospitals, especially those that maintain wards for tuberculous patients.

Significance of Skin Reaction in Children.—Stewart (1934) has published an interesting paper on the skin sensitiveness to tuberculin in primary tuberculosis. He reports the results of extensive roentgenologic and clinical investigations of 188 children ranging in age from 8 to 17½ years who gave positive skin reactions to the intracutaneous administration of 0.1 mg. of old tuberculin. In 132 children of this group he found either characteristic calcified hilus glands or Ghon tubercles or both, twenty-one showed slight or questionable calcification of hilus glands, eight showed only pleural thickenings and twenty-one showed negative chest plates. He states that clinically the entire group showed no symptoms of tuberculosis and could be distinguished from normal uninfected children only by means of the tuberculin test and roentgen examinations.

Tuberculin Surveys With PPD.—Long, Aronson and Seibert (1934) report on results obtained in tuberculin surveys with the purified protein derivative and a highly potent OT, respectively. This work was done, apparently, with an idea of determining the potency, uniformity, and general reliability of the new tuberculin. The results of their work may be partly summarized as follows:

1. They found that an initial dose of 0.00002 mg. of the purified protein derivative detects a high percentage of total positive reactors to OT with very few severe reactions. The remaining positive reactors to 0.01 mg. and 1.0 mg. of highly potent OT are detected by a dose of 0.005 mg. of PPD.

2. They recommend that PPD be incorporated into sterile lactose tablets in quantities of 0.0002 mg. and 0.05 mg. per tablet for convenience in the preparation of ten first or second doses to be used in tuberculin tests.

Park, Kereszturi, Mishulow (1933) also point out the superiority of the tuberculin test over the x-ray as an aid in the diagnosis of

primary tuberculosis. The roentgenologic findings are of great value, however, in the diagnosis of the reinfection or adult type of tuberculosis.

EFFECT OF AGE UPON DILUTED TUBERCULIN.—In regard to the effect of age upon *diluted* tuberculin Aronson says that Mantoux as early as 1909 noted that its potency decreased on standing and that Okell found that a 1:1,000 dilution of OT shows a diminution of potency, if kept at room temperature, of 40 per cent within ten weeks, and 60 per cent within one year. Aronson has observed that a 1:10 dilution of OT, incubated at 37° C., shows a marked loss of potency within seven to ten days and is practically inactive after several months. Undiluted tuberculin or the purified protein derivative incorporated with beta lactose in tablet form is quite stable.

SYSTEM OF GRADING MANTOUX TESTS.—Aronson (1934) employs an initial skin test dose of 0.00002 mg. of PPD, reads the results forty-eight hours later and reinjects all who are negative with 0.005 mg. of PPD. These doses of the purified protein derivative correspond to 0.01 mg. and 1.0 mg. of OT, respectively.

The system of grading which he has finally adopted is a slight modification of the one previously recommended by the National Tuberculosis Association. He says that the new system of grading enlarges the two-plus range and thus takes in some that were formerly graded three-plus. The new classification of tuberculin reactions is as follows:

+	Some redness and an area of edema more than 5 and less than 10 mm. in diameter.
++	Area of redness and edema 10 to 20 mm. in diameter.
+++	Area of redness and edema exceeding 20 mm. in diameter.
++++	Marked redness, edema and an area of necrosis.
Doubtful reactions	These show redness and traces of edema measuring 5 mm. or less in diameter. Redness is always regarded as of less significance than edema.

Long (1934) states that during the past twenty-five years there has occurred a reduction in the incidence of tuberculosis in the United States. He suggests that the antituberculosis campaign which has been carried on for many years is largely responsible for this reduction in the incidence of the disease.

Incidence of Tuberculosis Determined at Autopsy.—In 1917 Opie reported evidence of tuberculosis in 8.3 per cent of infants dying within the first two years of life, 44 per cent in children dying between the ages of two and ten years, 66.7 per cent for ages ten to eighteen years, and 100 per cent for those dying at later ages. These percentages include many cases of healed tuberculosis as well as cases that showed activity. In 1927 Opie and Aronson studied material from 169 bodies that showed what appeared to be healed lesions. By means of guinea pig inoculation they found that 20 out of 29 apparently healed lesions contained living tubercle bacilli.

In a later discussion of the epidemiology of tuberculosis, Opie (1932) calls attention to the continued high incidence of tuberculosis in Philadelphia and to the appalling number of serious tuberculosis infections revealed by anatomical studies. He states that such studies made in St. Louis and Philadelphia indicate that one out of every six white adults who die from diseases other than tuberculosis have partially caseous lesions at the apices of one or both lungs. He seems to interpret the diminution of the death rate from tuberculosis as indicating that the infection is now following a more benign course than formerly. Opie points out that the skin test is the most delicate method of detecting infection, although it does not differentiate between latent and manifest tuberculosis.

ROBERTSON'S ANATOMIC STUDIES.—On the other hand, the studies of Robertson (1933) and also of Terplan (1934) apparently support the statement of Long that the incidence of tuberculosis is less than it was twenty-five years ago. Robertson reviews the series of 3,306 autopsies done at the Mayo Clinic over a period of six years (1926-1931) to determine the incidence of tuberculosis. He found tuberculous lesions present in 2,064, or 62.43 per cent, of the bodies examined. As a result of histological studies he concludes that the lesions in 1,725 of these cases were healed and that there is evidence of active tuberculosis in 134, or 4.05 per cent. The incidence of active tuberculosis for the various age groups is interesting. For the first three decades of life it was 2.94, 6.71 and 6.84 per cent, respectively. For the decades between 30 and 70 years it was 3.78, 3.17, 4.47, and 3.99 per cent, respectively.

Terplan reports the anatomical incidence of tuberculosis among 312 white children whose ages are between one month and six years as 4 per cent; in 52 individuals ranging in age from seven to eighteen years, it is 20 per cent; while for the age groups twenty to forty and forty-one to eighty years, it is 60 and 95.7 per cent, respectively. Terplan calls attention to the close agreement between the incidence of 20 per cent having tuberculous lesions determined at autopsy for the age group seven to eighteen years and the 20 to 25 per cent positive reactors to tuberculin (Mantoux test) among the school children of Buffalo. It should be remembered that the majority of the anatomical tuberculous lesions were healed. In this study Terplan found some primary lesions in all age groups. In discussing this report Long remarks that Terplan has offered definite evidence that adults who escape primary tuberculosis infection in childhood are not likely to break down with massive primary tuberculosis as was formerly thought to occur. Apparently primary tuberculosis in adults tends to pursue a course similar to that observed in children.

Tissue Response to Various Fractions of the Tubercle Bacillus.

—The effect on the allergic animal of various fractions of the tubercle bacillus injected into the tissues has been studied extensively by Sabin and her associates. They report that the phosphatid fraction stimulates the formation of epithelioid and giant cells while the acetone-soluble fat causes the proliferation of connective tissue and blood vessels and also causes hemorrhages. The waxlike constituents stimulate fibroblast proliferation, and the carbohydrate fraction is both chemotactic and toxic for leucocytes. When the tubercular protein fraction is injected, both fever and the proliferation of plasma cells result.

The Necessary Factors for the Development of Tuberculin Allergy.—While one can produce antibodies for and render a guinea pig anaphylactic to tuberculin protein, it is interesting to note that tuberculin hypersensitiveness or allergy as previously described in this chapter does not result from the injection of tuberculin OT or any of the others described by Koch (Baldwin, 1910, Fleischner, Meyer and Shaw, 1919, Zinsser, 1931). The necessary factor for its production is the formation of a tubercle containing living or dead tubercle bacilli or a tuberculin that is antigenic; e.g., MA-100 within the tissues of a susceptible

animal or experimental conditions described by McJunkin and confirmed by Zinsser. McJunkin injected tubercle bacilli into the peritoneal cavity of infected guinea pigs and 24 hours later removed the peritoneal exudate, filtered and injected it into normal guinea pigs. A number of these normal recipients showed tuberculin hypersensitiveness when tested six or eight days later. Baldwin (1910) and Zinsser (1931) suggest that perhaps the train of events leading to the allergic state in tuberculosis may be described somewhat as follows:

First the tubercle bacillus lodges in the tissues and the body reacts to its presence by the formation of a tubercle. The action of the inflammatory tissue upon the bacteria, probably by means of enzymes, causes the liberation of an antigenic substance which is responsible for tuberculin allergy. It is only recently that one has been able to extract from the tubercle bacillus such an antigenic substance and no one has been able to prove that antibodies are involved. It is interesting to note that while tuberculin OT is devoid of skin sensitizing power, such tuberculins as MA-100 and TPT possess it. At first Seibert believed that OT and likewise PPD were not antigenic because of their small molecular weights and conversely MA-100 and TPT were antigenic because of their large molecular weight. Subsequent work has led Seibert (1941) to alter her views. She is now inclined to think that potency is inherent in one part of the tuberculin protein molecule while antigenicity is dependent upon another part of the same molecule. Apparently the work of Landsteiner (1935) (1938-1940), as well as the work of Stull and Hampton (1941), indicates that less complex substances than complete proteins can function as antigens. Stull and Hampton report that certain proteoses obtained from Witte's peptone are antigenic. It is possible, as suggested by the work of Landsteiner, that these substances function as haptens and unite with body proteins to form complete antigens. Seibert's work suggests that tuberculin may function as a hapten when it is antigenic.

A somewhat similar state of affairs exists in undulant fever. Fleischner, Meyer and Shaw (1919) as well as others have shown that while guinea pigs can be rendered anaphylactic by injecting suspensions of *Brucella abortus* and *melitensis*, skin sensitiveness does not develop unless active infection is present.

As a result of numerous investigations of the tuberculin, typhoidin, abortin and mallein reactions, which are of the tuberculin type, it appears that certain essential points of dissimilarity exist between protein anaphylaxis and bacterial allergy of the tuberculin type. An interesting review of the early literature along with important experimental data bearing upon this problem is included in an excellent paper by Fleischner, Meyer and Shaw (1919). For a more extensive discussion of Brucellosis the student is referred to a very interesting paper by Giltner (1934).

In 1921 Zinsser made an extensive study of bacterial allergy and anaphylaxis and summarized the points of difference between the two phenomena. Aronson (1933) investigated by means of tissue cultures the relation of the tuberculin reaction to anaphylaxis and the Arthus phenomenon. He confirmed the results of Rich and Lewis who found that tissue explants of bone marrow and spleen from tuberculous guinea pigs are killed by dilute solutions of tuberculin that are nontoxic for similar explants from normal animals. When he adds dilutions of horse serum to similar explants from guinea pigs sensitized to horse serum the tissue cells are not killed but grow as well as explants from normal guinea pigs used in control experiments. These findings are in harmony with the tissue culture studies of Meyer and Loewenthal (1927), cited by Aronson, although contradictory results are reported by others. The differences suggested by Zinsser have been tabulated by Dienes and Mallory (1932). Their table, with the addition of the difference suggested by Aronson (1933) and others, is shown in Table XVI.

POSSIBLE EXPLANATION OF APPARENT DISSIMILARITIES BETWEEN ANAPHYLAXIS AND TUBERCULIN ALLERGY.—It is generally agreed that the union of antigen and antibody is in some manner responsible for anaphylactic reactions. The first two points of dissimilarity between the latter and tuberculin allergy tabulated above would seem to indicate that the tuberculin reaction is not mediated by a similar mechanism. That such a conclusion is not warranted at the present time is indicated by the experimental results of Dienes and Schoenheit (1929, 1930) and of Dienes and Mallory (1932). The former report that when tuberculous guinea pigs are sensitized to crystalline egg albumen by injecting it directly into a tuberculous lesion and subsequently testing by injecting

TABLE XVI

	ANAPHYLACTIC TYPE	TUBERCULIN TYPE
Skin test	Immediate Transitory	Delayed Prolonged
Serum	Antibodies demonstra- ble Passive transfer pos- sible	Antibodies not demonstra- ble. Passive transfer not demonstrable
Results of intrave- nous injection	Acute shock	Delayed shock
Sensitizing antigen	Ordinary proteins Bacteria and some of their protein-contain- ing products	Bacteria, best living but also killed if in condi- tion to produce granu- lomatous tissue responses in tuberculosis. Tuber- culins MA-100 and TPT
Testing antigen	Proteins Carbohydrate fraction of bacteria (appa- rently the most effec- tive)	Bacterial proteins and pro- tein fractions only
Tissue culture re- sponse to antigen	Transplants of bone marrow and spleen from sensitized ani- mals not killed by antigen	Transplants of bone mar- row and spleen from tu- berculous animals killed by tuberculin

crystalline egg albumen intracutaneously, the resulting reaction is specific and of the tuberculin type, i.e., it is delayed and prolonged. When the sensitizing dose of egg albumen is administered elsewhere than directly into a tuberculous lesion, a skin test performed eight to ten days later with the homologous antigen results in an immediate and transitory or anaphylactic type of reaction.

Certain reports in the literature of exceptions to these results suggested to Dienes and Mallory that a time factor might be important. They, therefore, sensitized normal uninfected guinea pigs to egg albumen and egg globulin and noted the type of reaction when skin tests were made with the homologous antigen at four-, six-, eight-, and ten-day intervals following the administration of the sensitizing dose of antigen. They discovered that reactions obtained on the sixth day and earlier were of the tuberculin type, while the reactions obtained later were of the anaphylactic type. When skin tests were performed upon guinea pigs pas-

sively sensitized to egg albumen, they invariably observed the anaphylactic type of response.

Histological studies of the various skin reactions confirmed the gross findings. The anaphylactic reaction in passively sensitized guinea pigs is characterized by an immediate serous and polymorphonuclear exudate. In guinea pigs actively sensitized to egg albumen and tested on the sixth day the reaction is delayed. It appears in about six hours and persists forty-eight hours or longer. Microscopically the cellular reaction is predominantly mononuclear. When the actively sensitized pigs are tested on the eighth day or later the reaction is immediate and the cellular exudate contains a predominance of polymorphonuclear cells.

SIMILARITY BETWEEN IMMUNE RESPONSE AND TUBERCULIN ALLERGY.—The authors believe that the ratio of mononuclear to polymorphonuclear cells is a valuable criterion of differentiation between the two types of hypersensitiveness. They conclude that the tuberculin type of hypersensitiveness represents the first stage of the immune response to the introduction of any antigen within the tissues of a suitable animal. In tuberculous animals there is a fixation of this early stage for tuberculin protein so that skin reactions to tuberculin are always of the tuberculin type. In protein anaphylaxis, on the other hand, there is no fixation of the early stage, but, instead, the anaphylactic type supervenes after the sixth day.

The observation of Dienes that the injection of egg albumen and other protein antigens directly into a lesion causes in some manner subsequent skin reactions to the homologous antigen to be of the tuberculin type, whereas antigen injected elsewhere leads to the anaphylactic type of reaction, may be explained in part at least on the basis of the time factor.

If the egg albumen is injected directly into a lesion, its absorption may be delayed and the primary stage of the immune response therefore prolonged. Dienes' work suggests that dosage and other factors as yet unknown are involved. In his experiments where sensitizing injections of egg albumen were made away from the lesion it is interesting to note that skin tests were never done as early as the sixth day, hence the early stage typical of the tuberculin type of allergy was not detected. A careful reading of Dienes' papers reveals *many exceptions* and also nu-

merous *mixed reactions*: hence it is probable that host variation and other factors affect the results obtained.

Hanks (1935) confirmed the Dienes phenomena but he concluded that it is not necessary to inject the antigen into a tuberculous focus. Zinsser, Enders, and Fothergill (1939, p. 429) question Hanks' conclusion as to the latter point.

In addition to these results which suggest that tuberculin allergy in tuberculosis is mediated by an antigen-antibody mechanism, there are the observations of Rich and his colleagues as well as others before them that the tuberculous animals can be desensitized to tuberculin.

SUMMARY OF EVIDENCE INDICATING AN ANTIGEN-ANTIBODY MECHANISM.—The evidence which seems to indicate that bacterial hypersensitiveness due to infection is mediated by an antigen-antibody mechanism may be summarized as follows:

1. It results only from the presence of an antigenic complex, the whole bacterial cell, or from the introduction of a tuberculin such as MA-100 within the tissues of the body.
2. An incubation period comparable to that in anaphylactic sensitization is present.
3. The reaction is specific.
4. The early skin reaction in animals sensitized to egg white and other soluble proteins is similar to the tuberculin reaction in tuberculous guinea pigs.
5. In animals sensitized to the pneumococcus a typical tuberculin type of skin reaction can be demonstrated.
6. Desensitization to tuberculin can be accomplished.

One must admit that although the evidence just cited is strongly suggestive it cannot be regarded as proof that antibodies participate in the tuberculin skin reaction observed in tuberculous animals.

Aronson (1933) calls attention to a number of other explanations that have been offered by various investigators. He says that Bordet believes that the reaction may be due to an increased affinity of the tissues of a tuberculous animal for tuberculin; Koch assumes that tuberculin contains a necrotizing substance that is especially toxic for the white cells of tuberculous animals; Babes and Proca assume that it is a summation effect due to tuberculin

and a similar substance present in the tuberculous lesion; Selter regards tuberculin as a specific irritant for tuberculin-sensitive cells and assumes that its action is catalytic in that it promotes but does not participate in the reaction. The antigen-antibody theory was suggested by von Pirquet and Schiek in 1903 and is tentatively accepted by Zinsser, Enders, and Fothergill (1939). If antibodies do not participate in the reaction, then to us the most plausible alternative would be to accept the hypothesis that the tissues acquire the property of reacting directly with tuberculin without the participation of antibody.

The differences in the severity of tissue reactions (pointed out by Aronson and others) in tuberculin allergy and protein anaphylaxis, respectively, do not, in our opinion, invalidate the concept that the tuberculin reaction is mediated by an antigen-antibody mechanism. These results might be interpreted as indicating one or more of the following:

a. A qualitative or quantitative difference in antibodies which leads to a greater degree of sensitization in tuberculin allergy than in protein anaphylaxis. Under such conditions the meeting of the antigen (tuberculin) and its antibody might produce either a more profound physical change than in protein anaphylaxis or liberate more histamine or perhaps some substance with greater toxicity than is liberated in protein anaphylaxis.

b. The site of the reaction in tuberculin allergy may be intracellular, while in protein anaphylaxis it may occur at the cell surface. Such a difference in the field of operation might explain the difference in toxicity.

7. According to Seibert a tuberculin preparation (SOTT) was able to inhibit the precipitin reaction when tubercle protein and its homologous antiserum were brought together. The tuberculin (SOTT) had a molecular weight of 3,800. It apparently acted as a hapten.

Questions Raised by Antigen-Antibody Concepts.—For the present we are accepting the theory that the reaction of antigen and antibody is an important factor in tuberculin hypersensitivity. When one takes this stand he is confronted immediately with questions relating to the nature of the sensitizing and reacting substance, the site of antibody formation, the principal tissues in-

volved in the reaction, how antigen and antibody elicit the delayed response and the relationship of tuberculin allergy to immunity.

While it is true that most of these questions cannot be answered, yet it is possible to state a few experimentally derived facts that seem to fit into the complex picture and which may be regarded as partial answers to some of these questions.

In regard to the nature of the sensitizing substance responsible for tuberculin allergy, it seems that either the entire tubercle bacillus or an antigenic tuberculin must be present in the tissues of the body for sensitization to occur. Zinsser believes that the sensitizing antigen is a nucleo-protein whose antigenic property is destroyed during attempted extraction. This is apparently confirmed by Seibert's work (1941).

Antigenic Factors Present in Acid-Fast Bacteria.—Typical skin reactions are elicited by either large or exceedingly small doses of tuberculin or by large doses only of bacterial protein from other members of the acid-fast group. This suggests that the tubercle bacillus contains an antigenic fraction common to the timothy bacillus and other members of the acid-fast group.

On the other hand, Menzel and Heidelberger (1938) have isolated several fractions of proteins that differed chemically and serologically in each of the following acid-fast bacteria: human, bovine and avian tubercle bacilli and the timothy-grass bacillus.

In regard to the site of antibody formation one has what appears to be only a few alternatives, the tuberculous lesion itself, the reticulo-endothelial and other tissues of mesenchymal origin, and the epithelial tissues. It has been generally assumed that antibodies are produced by certain tissues of mesodermal origin.

Epithelium as the Site of Antibody Production and Antigen-Antibody Reaction.—Since Dienes (1933) has presented evidence that in tuberculin skin allergy the cells of the epithelium rather than the subepithelial tissues are the primary sites of the reaction, and since all attempts at passive transfer of antibodies have failed, one is faced with the possibility that if antibodies are involved in the skin reaction, they may be produced and retained by the cells of the epithelium and that the systemic reaction is due to antibodies produced by other tissues of the body. This is suggested also by the tissue culture work of Rich and Lewis (1927). They report

that transplants of bone marrow and spleen from tuberculous animals are killed by dilutions of tuberculin that have no effect upon similar cultures from normal animals. Furthermore Dorset, Henley and Moskey (1927) obtained a fraction of tuberculin which produces severe and fatal systemic reactions in tuberculous guinea pigs but is inert when administered intracutaneously. In the systemic reaction the tissues immediately adjacent to the lesions give the greatest allergic response.

Studies of Corper and Cohn.—These authors compare tuberculo-anaphylaxis and tuberculo-allergy. The former was produced by injection of tubercle protein while the latter was produced by infection. The differences they found between the two are:

1. Tuberculo-anaphylaxis symptoms could be elicited only when the shocking dose was given intravenously, whereas tuberculo-allergic intoxication could be induced by intravenous, subcutaneous, or intraperitoneal injections.

2. The symptoms of tuberculo-anaphylaxis were the typical ones of all protein anaphylaxis, whereas in tuberculo-allergic intoxication the symptoms were slow to develop, coming on in from a few hours to seventy-two hours, and did not resemble those of anaphylaxis.

Corper and Cohn also report that histaminase had no retarding or neutralizing effect on either tuberculo-anaphylaxis or tuberculo-allergy.

From the preceding discussion of tuberculin and other bacterial allergies, the following general conclusions can be drawn:

1. That tuberculin allergy develops from infection or vaccination with the tubercle bacillus or with an antigenic tuberculin.

2. The allergic state apparently results from the effect upon the tissues of some diffusible substance formed within a tubercle, presumably by the action of certain cellular enzymes within the lesion, upon the tubercle bacilli present.

3. The safest method of detecting tuberculin allergy is by the intracutaneous administration of small doses of tuberculin. It has been demonstrated that relatively large doses may give nonspecific reactions.

4. Allergy does not persist long after recovery.

5. It may be temporarily removed by desensitizing the patient or the same may occur as a result of massive infection.

6. The reaction may be suppressed during an attack of an acute exanthematous disease or during labor and the puerperium.

7. In some 3 to 5 per cent of the cases of miliary tuberculosis the reaction to tuberculin OT is absent.

8. The typical tuberculin reaction is of the delayed type and persists for several days. It is characterized by edema, more or less hyperemia and infiltration. Microscopically there is an initial neutrophile response followed by a preponderance of mononuclear over polynuclear cells. The cells are for the most part of vascular origin.

9. A similar reaction is demonstrable in protein anaphylaxis when skin tests are made on the fourth to the sixth day after the sensitizing dose is administered. When skin tests are made after the sixth day the reaction is immediate and of the anaphylactic type. The latter reaction is characterized by hyperemia, wheal formation, and an exudate containing a preponderance of neutrophils.

10. While antibodies have not been detected in tuberculin allergy, there is some evidence which suggests that the tuberculin reaction is mediated by an antigen-antibody mechanism.

11. It is suggested by Dienes that in tuberculosis there is a fixation of the early anaphylactic type of response.

12. In public health work the tuberculin test is of value in screening out of a large group of individuals for further study, those who have healed, potentially active, or active lesions of tuberculosis.

13. One cannot differentiate between primary and reinfection types of tuberculosis by means of the tuberculin test.

14. Likewise the severity of the reaction cannot be correlated with the type of lesion present.

15. Corper and Cohn report that the symptoms of tuberculo-allergic intoxication are different from those of tuberculo-anaphylaxis. They also state that histaminase does not prevent or modify the symptoms of either.

16. The purified protein derivative of tuberculin prepared in accordance with the recommended procedure of Seibert is generally regarded as superior to OT.

17. While anaphylactic sensitization can be produced with killed suspensions of the typhoid and abortus organisms, skin allergy is produced in experimental animals only when living organisms are injected and infection established.

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CHAPTER XXVII

THE SIGNIFICANCE OF ALLERGY IN TUBERCULOSIS AND A FEW OTHER DISEASES

Immunity in Tuberculosis.—As a result of extensive investigation it has been definitely established that the resistance of susceptible animals such as man, rabbits, guinea pigs, etc., to infection with the tubercle bacillus can be increased by infection with either virulent or avirulent human or bovine strains and also to a moderate extent by the injection of a suspension of killed tubercle bacilli.

Numerous anatomical studies of primary and reinfection types of tuberculosis offer abundant evidence that partial immunity results from infection with the tubercle bacillus. The primary or childhood type of tuberculosis occurs when the organisms lodge in a body that is neither allergic nor immune. In primary pulmonary tuberculosis the original lesion may be anywhere in the lung, no allergic symptoms are detectable and the regional lymph glands soon become involved. In the reinfection or so-called adult type of pulmonary tuberculosis the lesion is as a rule in the apex of one or both lungs, allergic symptoms may develop and the regional lymph glands are not involved because the tissue immunity, due to the first infection, localizes the organisms and limits the pathway of spread largely to the bronchi. For a more comprehensive discussion of the occurrence and significance of the various types of tuberculosis the student is referred to papers by McPhedran and Opie (1933), McPhedran (1933), Hetherington (1933) and Opie (1933). At the present time there is some controversy over the mechanisms involved and the rôle which allergy plays in acquired immunity to the tubercle bacillus. Krause (1910, 1916) made an extensive study of tuberculin allergy and immunity and suggested that tissue hypersensitiveness may play an important rôle in immunity.

It will be recalled that in Chapter VII the mechanism of acquired resistance to infection is discussed. According to Cannon,

Rich, and their respective colleagues, as well as a number of other investigators, certain factors of primary importance can be determined. Cannon et al., working with staphylococci, and Rich et al., studying dermal pneumonia in rabbits, report that when virulent organisms are introduced into the tissues of normal animals they not only undergo multiplication but are disseminated rapidly to various places remote from the point of inoculation. In immune animals the organisms remain localized although a certain amount of multiplication occurs. Apparently the presence of antibodies in the tissues causes the organisms to grow in chains or in clusters in the manner analogous to that observed in cultures grown in immune serum. The organisms not only tend to adhere to each other but they also stick to the tissue cells. Rich says that such phenomena may be observed before evidence of inflammation appears. He challenges the concept of Zinsser, Krause and many others that acquired resistance is due largely to allergy and the allergic inflammatory response. Rich grants that the pouring out of an inflammatory exudate after the bacteria have been localized is of value in diluting toxins or toxic substances, and that neutrophils and macrophages aid in destroying and removing bacteria, but he regards acquired resistance as consisting mainly of two factors: (1) the mechanism by which the organisms are retained at the point of entry and (2) the creation of an environment in which they are less able to survive than in the normal animal. He and his colleagues have shown that rabbits passively immunized to type I pneumococcus are highly resistant but not allergic to the organisms. As a result of this and other experimental studies he concludes that bacterial allergy and immunity can be definitely separated from each other. He and McCordock feel that this is especially true in human tuberculosis largely for the following reasons:

1. Krause and Willis planted tubercle bacilli into areas of tuberculin allergic inflammation and observed that the organisms did not remain localized. The results led them to conclude that immunity is reduced at the site of an inflammatory tuberculin reaction for at least four days.

2. Immunization with dead tubercle bacilli produced an intensive allergic state but only moderate immunity. Petroff and

Stewart (1925) state that "the local cutaneous allergic reaction in animals sensitized with dead bacilli varies in no way from that in infected animals."

3. Rich and McCordock cite the statement of Calmette (1927) to the effect that in immunizing with B.C.G. allergy may disappear for a while although acquired immunity persists.

4. They cite the experiments of Willis who found that animals immunized with a tubercle bacillus of low virulence developed tuberculin allergy but retained it for less than two and one-half years. After allergy had disappeared the animals were found to possess a pronounced degree of immunity when reinfected with virulent organisms.

5. In certain types of clinical cases of tuberculosis the administration of repeated and increasing doses of tuberculin results in both a disappearance of allergy and a beneficial effect upon the disease. This is especially true in certain types of skin and also ocular tuberculosis. They cite the observations of Hamman and Wolman that in a majority of cases treated with tuberculin a change for the better is associated with an increased tolerance of tuberculin.

6. In regard to the assumption that allergic inflammation stimulates fibrosis and the encapsulation of a tubercle, they call attention to the fact that connective tissue repair is a nonspecific process and, according to MacCallum, is in no sense a part of inflammation and may not necessarily follow a violent inflammation. That acute inflammation is not necessary for connective tissue repair is evident to anyone who studies the organization of a thrombus.

7. They call attention to the fact that the tubercle bacillus has little power to cause necrosis in the unsensitized body. After tuberculin allergy develops, it takes but a minute amount of tuberculin to produce extensive necrosis of tissue. In their opinion the killing of tissue within the body cannot be regarded as a defensive process.

8. The authors believe that in reinfection the mechanism of immunity operates to destroy the tubercle bacillus before the allergic reaction begins.

EFFECT OF ADMINISTERING VACCINE INTRAVENOUSLY.—Additional evidence which seems to support the theory of Rich and his col-

leagues that tuberculin allergy and immunity are not necessarily identical seems to be inherent in certain observations of Clawson (1934) and others. The former reports that animals receiving a heat-killed suspension of B.C.G. vaccine *intravenously* develop definite immunity to the tubercle bacillus without becoming perceptibly allergic to tuberculin as indicated by the Mantoux test.

Birkhaug (1933), on the other hand, holds to the opinion that allergy is an important and necessary factor in immunity to tuberculosis. He studied the immunizing property of B.C.G. vaccine for guinea pigs over a period of two years and reports that intradermal immunization yielded a more desirable and intense allergy as well as a greater percentage of relative immune animals than intraperitoneal immunization. In his opinion B.C.G. is incapable of producing progressive tuberculosis.

Experimental and Clinical Studies of Allergy and Immunity in Tuberculosis.—That these apparently conflicting opinions are not irreconcilable would seem to be apparent from a careful consideration of the following experimental studies of Smith (1933), Lurie (1936, 1939), Menkin (1938) and Cannon and Hartley (1938).

While the research discussed in the preceding pages of this chapter throws a great deal of light upon allergy and immunity in tuberculosis, there is inherent in them certain artificial and abnormal conditions with which the student should be made acquainted. The factors involved, which may cause one to acquire a misconception of what occurs in naturally acquired tuberculosis, have been clearly set forth in a paper by Theobald Smith (1933). He points out that in natural infection with the tubercle bacillus the entry through the mucous membrane of the respiratory or alimentary tract is not accompanied by mechanical injury to the tissue, such as results from hypodermic injection, and the dosage is limited to one or at most a few organisms as contrasted with relatively massive doses employed in experimental work. Furthermore the organisms involved in natural infection have a rate of multiplication and a metabolism that show the effect of a parasitic environment, whereas in experimental work the organisms have been acclimated to a saprophytic existence in artificial media and possess an accelerated rate of growth. Their metabolic products are perhaps essentially different from the organisms that have been growing under parasitic conditions. Some of the

conclusions which he holds as a result of an extensive study of spontaneous bovine tuberculosis and paratuberculosis may be summarized as follows:

SUMMARY OF THEOBALD SMITH'S STUDIES OF SPONTANEOUS BOVINE TUBERCULOSIS.—1. Whereas the concept of phagocytosis has been regarded as a one-sided affair, in reality one can only postulate that certain bacteria and cell types possess an affinity for each other. The results of such mutual attraction may vary from death of the host to partial or complete suppression of the parasite. In tuberculosis, paratuberculosis, leprosy, glanders, as well as in induced *Brucella* infections in guinea pigs, the bacteria associated themselves with cells of the connective and adenoid tissue. Their association leads to the development of the epithelioid type of cell. In typhoid and paratyphoid infections the bacteria have an affinity for endothelial leucocytes, while in diseases due to *Rickettsia* and in mouse septicemia the organisms exhibit a marked affinity for vascular endothelium.

2. In his opinion both allergy and immunity in tuberculosis result from the "discharge of diffusible products of the living bacilli and the continuous impact of these substances in minute doses upon the forerunners of the epithelioid cells throughout the system."

3. He observed that in spontaneous bovine tuberculosis the secondary foci exhibit definite evidence of having developed under the influence of an acquired immunity. In contrast with the primary lesions they are smaller, show a lessened tendency to necrosis, and contain giant cells.

4. The primary focus of epithelioid cells observed in spontaneously acquired bovine tuberculosis is not invaded by neutrophiles as in experimentally induced bovine tuberculosis. The neutrophiles do not seem to be involved in the initial stages of spontaneous tuberculosis.

5. He attributes a dual rôle to the focal lesions in tuberculosis. They are places where the bacteria multiply and places where the micro-organisms are being opposed by the host. The focus represents a compromise between the host and parasite.

6. In his opinion it is impossible to visualize all of the factors involved in the inner workings of the tuberculous focus because they are in a state of change. In the primary lesion of spon-

taneous bovine tuberculosis the early development of central necrosis masks the earliest phases. He assumes that the latter consist of the multiplication of the bacteria, the swelling of the cytoplasm of the local tissue cells, and then the development of central necrosis. The diffusion outward of products of metabolism of the tubercle bacillus leads to the formation of the epithelioid cell mantle and connective tissue capsule.

7. He points out that *immunity in tuberculosis tends toward a diminishing susceptibility instead of an increased activity on the part of the cells*. This results finally in the feeblest reaction, the formation of the giant cells. It is evident that this conclusion of Theobald Smith's is in harmony with the view of Rich and others that immunity and allergy are not necessarily identical.

THE IMMUNIZING VALUE OF NONPROGRESSIVE PRIMARY LESIONS.—Lurie (1933), in his experimental studies of contact tuberculosis, seems to have avoided many of the sources of error which have just been discussed. In 1930 he reported that cattle immunized with von Behring's bovovaccine were resistant for several months to the artificial introduction of fatal doses of tubercle bacilli of the bovine type. When, however, such immunized animals were stabled for twelve or more months with tuberculous cattle that were eliminating virulent tubercle bacilli, the immunized animals acquired tuberculosis. Such conditions of exposure to infection are to a certain extent comparable to the environment of a child in contact with tuberculous parents. Lurie also studied contact tuberculosis in guinea pigs. It was evident from these studies that the route of infection depended upon the relative intensity of exposure by the respiratory or alimentary channel. He determined that the reason crowding is so conducive to the spread of tuberculosis is the amount of virulent tubercle bacilli available for contagion.

In 1933 Lurie produced localized, nonprogressive infection in rabbits by inoculating them with living tubercle bacilli of the human type. When such rabbits were used as cage mates for normal rabbits, the latter died with contact tuberculosis. In order to ascertain the effect of such localized, nonprogressive tuberculosis upon the resistance of rabbits to contact infection with virulent bovine tubercle bacilli, Lurie exposed these infected

rabbits to cage mates that had been infected with virulent organisms. He determined by experiment that animals receiving 0.001 to 0.00001 mg. of highly virulent tubercle bacilli of the bovine type develop progressive lesions and eliminate virulent organisms. Normal animals were exposed to contact infection by placing them with cage mates thus infected. By means of a very clever experiment in which a special type of animal cage was used, he was able to show that normal animals and those having nonprogressing lesions acquire infection with virulent bovine tubercle bacilli under conditions in which the opportunity for alimentary infection is eliminated. The results of his experiments may be summarized as follows:

SUMMARY OF LURIE'S STUDIES ON ACTIVE IMMUNIZATION.—1. Of the normal rabbits exposed to cage mates infected with virulent bovine tubercle bacilli, 63.6 per cent contracted the disease within six months and 73 per cent within one year.

2. That the rabbits having a primary nonprogressive lesion gained some immunity from the primary infection is indicated by the fact that only 36.8 per cent of those exposed to cage mates eliminating virulent bovine tubercle bacilli contracted infection within six months and 60 per cent within one year.

3. Since 27 per cent of the normal rabbits and 40 per cent of the ones vaccinated with living human type tubercle bacilli escaped infection, it would seem that natural resistance plays a very important rôle in immunity to tuberculosis. Lurie* points out that vaccination saved only an additional 13 per cent.

4. His results show that "vaccination reduced the incidence, extent, and mortality of the disease, affected the route of infection, changed its pathological character, and retarded its progress. The disease acquired by vaccinated rabbits shared many characteristics with adult type tuberculosis in man."

LURIE'S INVESTIGATION OF THE MECHANISM OF IMMUNITY AND THE RÔLE OF ALLERGY IN TUBERCULOSIS.—Lurie (1936, 1939) has studied the mechanisms of resistance and the rôle of allergy in normal, tuberculous and vaccinated rabbits and guinea pigs. He introduced agar impregnated with tubercle bacilli in the tissues. He found that in normal animals the bacilli grew unhindered in the

*Lurie: J. Exper. Med. 58: 305, 1933.

acellular agar at a considerable distance from the phagocytic cells unless the tissue fluids were prevented from reaching them. This was in marked contrast to the inhibition of growth of the bacteria in vaccinated and tuberculous animals. Apparently the fluid from the tissues of normal animals penetrating the agar encouraged growth of the bacteria while the tissue fluid in vaccinated or tuberculous animals penetrated the agar and inhibited growth. This apparently demonstrated that humoral factors play a rôle in defense against tubercle bacilli.

Lurie also suggests that *in vivo* agglutination, as well as the deposition of a fibrin barrier thrown about the agar mass as a whole, tends to keep the bacteria localized in immune animals. It would appear that extracellular factors may be effective in limiting the spread of infection when small doses are introduced. When larger doses are introduced into vaccinated or tuberculous rabbits, there is a greatly intensified inflammation developed and the factors tending to localize the bacteria are overcome by the increased lymph flow which sweeps the leucocytes and agar particles containing bacteria into the regional lymph nodes more rapidly than in normal animals. This phenomenon has been reported by Freund and Angevine (1938) and others. Lurie found that immunity is effective in these vaccinated animals despite the lack of immediate fixation. This immunity is due not only to the humoral factors but to actively phagocytic mononuclear cells. The bacteria that are swept into the regional lymph nodes are fixed and destroyed by the humoral and tissue mechanisms.

Lurie found the mononuclear phagocytes much more effective agents in combating infection in vaccinated animals than the neutrophils. In fact he states that the mononuclear cells derived from vaccinated or actively tuberculous guinea pigs exhibit a greater *in vitro* phagocytic capacity for tubercle bacilli and carbon particles than mononuclears from normal animals.

Lurie also notes that bacteria are fixed locally more thoroughly in vaccinated or tuberculous guinea pigs than in vaccinated or tuberculous rabbits although the general immunity may not differ. He correlates this difference in local fixation with the differences he observed in the degree of allergic response and also the kind of fibrin networks laid down. He calls attention to the conclusion of Menkin (1938) that the amount of fixation of bacteria at the

point of infection is to a certain extent in proportion to the degree of local tissue injury, e.g., the staphylococcus produces a great deal of local tissue injury and is also fixed more extensively than other organisms producing less local necrosis.

Lurie states that the guinea pig develops more allergy to the tubercle bacillus than the rabbit. When tubercle bacilli are introduced into its tissues (agar-bacteria implantation) there is both a greater allergic response and a greater local fixation of bacteria. In the allergic inflammation of the guinea pig the fibrin network is finer, more compact, and there is a plugging and occlusion of the lymphatics. In the rabbit similarly treated, the fibrin network is coarser and the lymphatics remain open. These findings may account for the apparent contradictory conclusions drawn from experimental work mentioned earlier in this chapter. Some of the work on experimental allergy and immunity was done on rabbits and some on guinea pigs.

CANNON'S AND HARTLEY'S WORK ON ALLERGY AND IMMUNITY.—Cannon and Hartley rendered rabbits allergic to egg albumen and then injected a mixture of egg albumen and virulent pneumococci. The allergic inflammation did not protect the rabbits from a fatal pneumococcus infection. The organisms "grew profusely in the areas of allergic inflammation and spread evenly through the field of inflammation. While phagocytosis was moderately active, it did not modify the course of infection either in time or degree." This was in marked contrast to the phenomena observed in rabbits allergic to egg albumen but immune to the pneumococcus. In these rabbits the organisms grew profusely for a time but they became swollen, developed in isolated colonies, many became gram-negative and tended to remain localized. These experiments apparently show that, for the pneumococcus infection in rabbits, allergic inflammation without the aid of immunity factors such as antibodies cannot be regarded as a defensive mechanism.

DISCUSSION.—From a consideration of the results of Smith, Lurie, and Cannon and Hartley presented in the preceding paragraphs the following conclusions seem to be justified:

1. That as the body becomes more immune to the tubercle bacillus there is a diminishing tendency for any visible response. There is no evidence of allergy where complete immunity exists.

2. That the most important factors in the type of immunity (partial, i.e., not absolute) to the tubercle bacillus resulting from vaccination or infection are mononuclear phagocytes and humoral elements.
3. That in the tuberculin allergic inflammation in the guinea pig a more efficient mechanical barrier favoring localization is laid down than in the hypersensitive rabbit.
4. That within limits the degree of injury to the tissues in an area of allergic inflammation may affect the amount of local fixation of the infectious agent. Too large a dose of the bacteria may lead to a breakdown in the local mechanism of fixation and permit a more rapid spread to the regional lymph nodes in an allergic animal than in a normal one.
5. Allergic inflammation unsupported by specific immunity factors fails to prevent the escape of bacteria from the area of allergic inflammation.

It would seem from the above discussion that many of the apparent contradictory conclusions can be, in a measure, reconciled. In this connection a few other observations may be of interest. Teale (1935) is of the opinion that "hypersensitiveness is due to the state of the tissues which cannot deal with the specific antigen even when it is completely saturated with the homologous antibody." Woodruff and Willis (1939) conclude that there is a partial reciprocal relationship between the allergic state of infected guinea pigs and the number of bacteria found in the lungs. Corper (1940) has attempted to compare tuberculin allergy due to infection with anaphylactic hypersensitiveness to tubercle proteins. Apparently further work is indicated before it can be evaluated.

Effect of Primary Lesion in Children Upon Subsequent Infection.—At the present time there is considerable discussion as to the effect of initial or primary infection on subsequent tuberculous lesions in man. It is quite generally held that primary infection with the tubercle bacillus immunizes the individual and in part at least protects against later infections from exogenous sources. Myers and Harrington (1934) conclude from their study of a very large number of children that five times as many of those with initial positive tuberculin tests fall ill in later years with tuberculosis as do those who on first examination give

negative tests. In their opinion the primarily infected children offer a double liability in that many of them continue to harbor virulent organisms and all of them are allergic. When reinfection occurs the organisms are implanted in allergic tissue. The resulting reaction leads to destructive and progressive changes. Casperis, in discussing the work of Myers and Harrington, suggests that the children who show positive tuberculin tests on first examination constitute a group that has greater exposure to tuberculosis than the negative reactors and therefore, it might be expected that more of this group would develop the reinfection type of tuberculosis as a result of repeated exposure. Myers states, however, that in his series, contact was broken so far as possible immediately after the skin tests were done. It is difficult to compare these results with the animal experiments of Lurie since the primary infection in the rabbits was with the human type of tubercle bacillus which does not produce progressive tuberculosis in rabbits and hence there is no danger of adult tuberculosis arising from the flaring up of old primary foci such as may occur in children. The evidence at hand seems to show that mild primary tuberculosis confers a relative immunity as well as definite allergy upon an individual; that excessive exposure to virulent tubercle bacilli may break down this immunity; and that in such cases the allergic inflammation helps in that it assists in mobilizing phagocytic cells but is detrimental in that it plays a definite role in necrosis, extension of the inflammatory processes and general systemic reactions.

Different Opinions as to B.C.G. Vaccination.—For a great many years there has been a spirited debate over the question of active immunization against tuberculosis. There are at least four distinct views being presented at the present time. One group would not immunize at all but would depend entirely upon measures designed to prevent first infection; a second group would supplement such measures by immunizing all tuberculin-negative children with a killed vaccine; the third group would substitute B.C.G. for the killed vaccine; while the fourth group feels that vaccination should be viewed experimentally for fifteen or twenty years before it is generally adopted or rejected.

Those who would not immunize at all state that in their opinion immunization is at best a questionable practice, subsequent con-

tamination may occur and that the administration of the vaccine produces tuberculin allergy in the vaccinated children. This latter they regard as detrimental.

The second group is especially concerned over the possible increase in virulence of the B.C.G. organisms and the subsequent development of tuberculosis. Petroff and others have presented evidence supporting this view.

The third group, which favors immunization with an attenuated bovine tubercle bacillus vaccine such as B.C.G., maintains that the vaccine is safe and efficacious. They disregard the development of allergy, in fact many of those favoring this vaccine consider allergy as desirable.

The fourth group is made up of conservative individuals who do not desire to experiment upon their private patients. They constitute a very large percentage of group one who are willing to accept vaccination or any other method only after such methods have been tried and found successful over a fairly long period of time. In their opinion adequate data upon which a final conclusion can be based will come only after the present large number of experimentally immunized children are followed for several decades and their ultimate fate determined.

PARK'S STUDIES ON B.C.G.—An investigation of the effect of vaccination with B.C.G. on children from tuberculous families has been inaugurated in New York by Park, Kereszturi, Camille and Mishulow (1933). The results of their work may be summarized as follows:

1. They observed no evidence of an increase in virulence of B.C.G. when inoculated into a long series of rabbits and guinea pigs nor from residence in the human body.

2. They immunized 205 tuberculin-negative children by oral and 150 by parenteral vaccination and observed them for five years relative to the phenomenon of allergy and symptoms of clinical tuberculosis. Comparable control groups were included in their series. In the orally vaccinated group approximately 1 per cent died from tuberculosis as compared with 3.2 per cent of deaths in the control group. While no deaths occurred among those parenterally vaccinated, it is interesting to note that the death rate was only 1.4 per cent among the tuberculin-negative

controls and 3.2 per cent among the children with positive initial Mantoux reactions. In regard to the efficiency of oral vaccination, Park states that if, following its administration, the development of a positive tuberculin test is to be the criterion of its efficiency, then only 20 to 40 per cent of the orally vaccinated children can be regarded as effectively vaccinated. They noted that allergy following vaccination does not last, as a rule, longer than two or three years.

3. Park et al. did not find B.C.G. vaccination to improve the general health nor to protect against other infectious agents as claimed by Calmette.

4. Bogen in discussing the paper presented by Park et al. calls attention to the value of the tuberculin test in diagnosing active, primary tuberculosis in children. Since B.C.G. immunization leads to the development of tuberculin allergy, it is obvious that the test cannot be used in vaccinated children to diagnose active tuberculosis. He regards this as one objectionable feature of vaccination.

5. Park regards the results they obtained as not conclusive at the present time. The student will find from a perusal of the literature that aside from the work just discussed, the majority of the reports of the use of B.C.G. vaccine represent experiments that are either uncontrolled or only partially controlled. Such are the studies of Wallgren (1934) covering a short series of vaccinations in Sweden. His conclusions relative to virulence and immunizing value of B.C.G. are in accord with the conclusions of Park and his colleagues. Miller (1940) likewise says that neither B.C.G. nor any other preparation has been proved to be of widespread usefulness. The new "Vole tubercle bacillus" vaccine of Wells and Brooke (1940) has been discussed in an earlier chapter. It is apparently superior to the B.C.G. vaccine as an immunizing agent in guinea pigs.

Other Bacterial Allergies.—*Brucellosis.*

Reference has been made earlier in this chapter to the typhoidin, mallein and abortin reactions. The latter is used to a limited extent as an aid in the diagnosis of undulant fever in man although it has not replaced the agglutination test. These reactions are all of the tuberculin type. Schoenholz and Meyer (1927) carried

out extensive studies on the purification of abortin. Since a satisfactory synthetic medium has not been devised, they proceeded to fractionate cell solutions prepared from whole bacteria by acetic acid precipitation. They report that the acid-precipitable fraction of abortin is as active as the original solution. The active principle is destroyed within twenty-four hours by tryptic digestion. Schoenholz and Meyer could produce neither specific increased resistance nor allergy by immunizing guinea pigs with killed suspensions of *Br. abortus* although the agglutinin titer for the antigen was definitely increased. They conclude that allergy results only when active infection is present. Theobald Smith reports that immunization of cattle with a suspension of killed *Br. abortus* confers only partial and transient immunity. He finds that a vaccine composed of living organisms is superior to the former. For additional information relative to Brucellosis the student is referred to a few supplementary references and to an excellent monograph by Giltner (1934).

STREPTOCOCCUS ALLERGIES.—In view of the prevalence and frequent chronicity of streptococcus infections it is not surprising that many individuals are allergic to the nucleo-protein fraction of scarlet fever streptococci. Dochez and Stevens (1927) carried out extensive studies on streptococcus allergy in guinea pigs and rabbits. They interpret their results as apparently favoring the theory of Bristol (1926) that the rash and clinical symptoms of scarlet fever may be due to the development of allergy to scarlet fever streptococci or their products.

RHEUMATIC FEVER.—The etiology of rheumatic fever is not definitely established at the present time. Clawson reports isolating streptococci from a high percentage of blood cultures of rheumatic fever patients and of producing typical pathological lesions in experimental animals. Herry (1914) and more recently Swift and his colleagues explain the symptoms and clinical course of rheumatic fever upon the basis of streptococcus allergy. An excellent brief summary of this work is given by Zinsser (1931, 1939).

PNEUMOCOCCUS INFECTION.—McBroom and Schlesinger report that there occurs an early stage of hypersensitiveness to pneumococcus nucleoprotein simultaneously with the early stage of resistance.

THE SHWARTZMAN REACTION.—In 1928 Shwartzman described a new phenomenon of local skin reactivity to certain bacterial cultural filtrates. Subsequent studies (1930, 1934) have revealed many interesting facts regarding the reaction. In his original work he reported that if one injects a small amount of a culture filtrate of *E. typhosa* into the skin of a rabbit, there is very little or no local response. If, twenty-four hours later, one administers to the same rabbit a small amount of the filtrate intravenously, one observes a severe hemorrhagic reaction developing at the site where the filtrate was injected into the skin twenty-four hours previously. Shwartzman concludes that the filtrate contains skin *preparatory* and *reacting* factors. He has shown that the preparatory effect is not due to trauma, local reticulo-endothelial blockage, increased permeability of the capillaries or to inflammation. In Shwartzman's opinion the vulnerability is due probably to a disturbance of cell function. The susceptibility disappears completely in forty-eight hours. The preparatory and reacting factors are not present in the filtrates of all bacteria nor in crystalline egg albumen or horse serum. The preparatory factor in the filtrate of one kind of bacteria, e.g., *E. typhosa*, can render the skin or organ or a rabbit susceptible to the reacting factor of another bacterial filtrate; e.g., of the meningococcus. When a potent filtrate is treated with the homologous immune serum, both factors are neutralized in multiple proportions. In this as well as in their synergistic effects they resemble toxins. Because of the short incubation period (twenty-four hours), the inability to transfer susceptibility passively, and the phenomenon of synergism noted above, Shwartzman concludes that his reactions are not to be classified as anaphylactic in nature.

Apitz (1935) has made a rather extensive study of the Shwartzman reaction and describes a generalized reaction elicited by intravenous rather than subcutaneous injections comparable to the classical Shwartzman phenomenon. He has also made chemical studies of the Shwartzman-active substances. He finds the active substances are contained in two different chemical fractions of bacterial substances. The so-called "nucleoprotein" fraction is active and is derived from bacteria by autolysis or extraction. There is also an alcohol-precipitable biuret-negative fraction. It

is unstable, precipitable with small volumes of alcohol, and Apitz says it is not the same substance as the type-specific carbohydrates.

Stolyhwo (1936) reports that the active Shwartzman substance is concentrated in the urine of typhoid patients. He thinks the active substances may be responsible for severe intoxications in typhoid fever.

FREI TEST IN LYMPHOGRANULOMA INGUINALE.—DeWolf and Van Cleve (1932) give an excellent discussion of lymphogranuloma inguinale and of the intradermal test suggested by Frei (1925) for its diagnosis. It seems to be established that the disease is venereal and caused by a filtrable organism which according to Tamura (1934, 1935) can be cultivated *in vitro*. According to DeWolf the antigen for the original Frei test is prepared by making a one to ten dilution of pus obtained from a suppurating gland. Physiological saline is used as a diluent. The preparation is then heated at 60° C. for two hours on one day and one hour on the next day. It is then cultured for sterility, and if no bacterial growth is obtained, the material thus prepared is used as an antigen in 0.1 c.c. amounts for intradermal injection into patients suspected of having the disease.

DeWolf and Van Cleve state that cutaneous allergy develops quite early in the disease and persists for a long period after recovery. A positive skin reaction develops at the site of the intradermal injection within twenty-four hours and persists for several days. The reaction appears as an inflammatory papule and resembles a positive tuberculin reaction. The authors regard it as of great diagnostic value.

Tamura (1934, 1935) reports the successful cultivation of the virus in a medium consisting of filtered Tyrode's solution and bits of sterile rabbit or guinea pig tissue as suggested by Maitland, Laing and Lythe (1932) in their cultivation of vaccinia virus. Tamura reports that in cultures of the virus in this medium the supernatant fluid becomes cloudy and that it can be heated and used as an antigen in the Frei test and also in immunization experiments.

Connor, Levin and Ecker (1937) report that the Frei test is specific for lymphogranuloma inguinale and that a positive test has been obtained as long as 39 years after the infection.

Summary and Conclusions.—

1. Evidence is offered which seems to show that tuberculin allergy and immunity are not identical and that the former may be detrimental to the host.

2. A summary of Theobald Smith's excellent discussion of experimental tuberculosis is given.

3. Primary tuberculosis confers a relative immunity and also allergy upon the individual. There is a difference of opinion as to whether the detrimental effects of the latter outweigh the beneficial effects of the former. In any event the primary lesion represents a focus of virulent organisms that may ultimately lead to endogenous reinfection.

4. Relative immunity and allergy are apparently produced by immunizing with suspensions of either killed or living attenuated tubercle bacilli. The route of immunization may determine the degree of allergy that occurs.

5. The work of Park, Kereszturi and Mishulow on the effect of vaccination with B.C.G. on children from tuberculous families is presented. From these studies and those of Lurie it is evident that natural resistance is an important factor in prevention of tuberculosis. They conclude that B.C.G. is safe but confers relatively little immunity and considerable allergy when administered to children. Park regards the results as inconclusive.

6. In our opinion, the problem is still in the experimental stage and should be so regarded until more carefully controlled work similar to that of Park et al. is completed.

7. Allergy to streptococcus nucleo-proteins is observed not infrequently in man. Ando et al. have shown that this is a possible source of error in interpreting the Dick test unless a purified toxin is employed as the indicator antigen.

The clinical symptoms and cutaneous manifestations of scarlet fever are regarded by Dochez and Sherman as manifestations of bacterial allergy. There are also a number of investigators who regard rheumatic fever as a manifestation of streptococcus allergy. At the present time the prevailing opinion as to the etiology of scarlet and rheumatic fevers, respectively, is that the former is a toxemic and infectious disease caused by certain hemolytic streptococci, while the etiology of the latter, rheumatic fever, is as yet either undetermined or not generally agreed upon.

8. Shwartzman discovered that the culture filtrates of certain kinds of bacteria, e.g., *E. typhosa*, *Esch. coli*, the meningococci, streptococci and a few other bacteria, contain what he designated as a skin preparatory and reacting factor respectively. If a small amount of such a filtrate is injected into the skin of a rabbit, and twenty-four hours later an intravenous injection of the same or any other filtrate that contains the reacting factor is administered, there develops a severe hemorrhagic reaction at the site of the primary skin injection. In Shwartzman's opinion the phenomenon is due to a local disturbance of cell function produced by the filtrate. He reports that the skin preparatory and reacting factors of a filtrate may be neutralized by the addition of its homologous immune serum. A few additional observations are reported.

9. Allergy in lymphogranuloma inguinale is mentioned and a skin test as originally described by Frei is described and discussed. The etiological factor is apparently a filtrable virus which Tamura reports having cultured in vitro. According to Tamura the supernatant fluid of positive cultures can be heated and used successfully in skin testing or in immunization. If his results are confirmed, a commercial antigen should be available in the near future.

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CHAPTER XXVIII

HYPERSENSITIVENESS

Human Idiosyncrasies

Clinical Allergies.—It is generally recognized, at the present time, that a large number of individuals (variously estimated at from 7 to 15 per cent) are abnormally sensitive to one or more protein or nonprotein substances. In the group of clinical allergies one places hay fever, asthma, urticaria, eczema, contact dermatitis, serum disease and drug allergy. Rackemann and others suggest that migraine, angioneurotic edema, erythema multiforme and perhaps epilepsy are manifestations of hypersensitiveness.

REACTIONS OCCUR IN LOCAL SHOCK ORGANS.—In human idiosyncrasies the reactions tend to be local rather than general. This has led Doerr to introduce the term "shock organs" to indicate the organs or tissues in which evidence of hypersensitiveness appears. Thus in vasomotor rhinitis the "shock organ" is the mucous membrane of the nose, in asthmas it is the lungs, while in eczema, contact dermatitis, etc., it is the skin. Duke and others report numerous cases of allergy in which the "shock organ" is the gastrointestinal tract. Coca calls attention to the fact that not all of the shock organs are affected in every atopic patient. Many hay fever patients do not have asthma and conversely some patients with asthma do not have hay fever or urticaria.

INHERITANCE FACTORS IN ATOPY.—Cooke and Vander Veer seem to have established an hereditary factor for the clinical allergies other than serum disease and contact dermatitis (poison ivy, etc.). Their data indicate that the capacity to become allergic is an inheritable characteristic which follows the Mendelian principles. There is no evidence to indicate that hypersensitiveness to a specific substance is inherited; instead only the *capacity* to become allergic appears in the offspring. A parent may suffer from hay fever due to ragweed pollen while the offspring may develop eczema from eating eggs. There is no unanimity of opinion at present as to whether the hereditary character is dominant or recessive. Such

forms of hypersensitiveness are designated by Coca as atopy. Zinsser, Enders and Fothergill (1939) and Coca (1931) state that two factors determine the development of atopic hypersensitiveness; i.e., contact and the inherited capacity to become sensitized.

Reagins Not Demonstrable in All Cases of Allergy.—While specific antibodies, which Coca calls *reagins*, are present in the blood of all asthma and hay fever patients who give positive skin tests, specific reagins have not been found in those suffering from contact dermatitis, serum disease or drug allergy although there is evidence which suggests that they, too, are mediated by an antigen-antibody mechanism. Zinsser (1931) bases his belief that all human idiosyncrasies and anaphylaxis are mediated by similar if not identical mechanisms upon the following facts: The idiosyncrasies are specific, are frequently accompanied by specific skin reactions, are alike regardless of the kind of exciting agent involved, and are often amenable to desensitization. One might add also that in experimentally produced contact dermatitis, in serum disease, and in many cases of drug allergy, the idiosyncrasy develops after an incubation period. The assumption that antibodies do not participate in these three types of allergy is based upon negative results where passive transfer has been attempted. We agree with Zinsser that such negative findings do not now constitute adequate reasons for assuming a different mechanism from that demonstrable for most of the atopies and for anaphylaxis.

Loveless (1940) reports the existence of two antibodies in the serum of hay-fever patients. One is the reagin and the other is an antibody that binds and neutralizes the homologous antigen.

PASSIVE TRANSFER OF REAGIN AND THE P-K REACTION.—In 1919 Ramirez reported that a patient transfused with blood from a donor sensitive to horse proteins became hypersensitive to the same exciting agent. Doerr cites a number of examples of passive sensitization following transfusion from allergic donors. It is generally agreed, however, that such cases are extremely rare. In 1921 Prausnitz and Küstner discovered that the serum of many hypersensitive individuals, when injected intradermally into a normal person, renders the local area injected hypersensitive to the same exciting agent to which the donor is sensitive. Küstner was sensitive to fish. He demonstrated that his serum contained anti-

bodies or reagins for fish proteins by injecting a small amount of his serum intracutaneously into the forearm of a nonsensitive person. A few hours later, when the area thus injected was tested with an extract of fish proteins, a positive skin reaction occurred. This phenomenon, which indicates the presence of reagins in serum, is called the Prausnitz-Küstner or P-K reaction. Coea and Grove state that only about 80 per cent of normal skins are receptive.

In 1937 Cowie reported upon a series of individuals who were clinically sensitive but skin negative to certain food allergies. He stated that the intradermal injection of such a patient's own serum into his skin rendered the injection site sensitive to the allergin. This he called autopassive transfer. Martin (1940) was unable to confirm Cowie's conclusions. On the contrary he states that the patient's own serum has an inhibitory rather than an enhancing effect.

Sensitization Through Alimentary and Respiratory Tracts and Through Placenta.—Walzer (1927) made use of the Prausnitz-Küstner reaction to ascertain whether the exciting agent is absorbed through the mucous membrane of the intestine. He injected serum from an egg-sensitive patient and from two fish-sensitive patients intracutaneously into the forearms of nonsensitive individuals. The following morning he fed the recipients egg and fish, respectively, and observed redness and swelling develop at the site of the serum injections.

In 1936 Gray and Walzer reported on the absorption of undigested peanut protein following oral and intraduodenal administration. They have also found (1940) this protein to be absorbed from the rectum.

Gay and Chant passively sensitized to ragweed an area in the skin of the forearm of a nonsensitive individual and after an adequate incubation period had elapsed they injected ragweed pollen extract intradermally into the opposite forearm. This caused a positive reaction characterized by erythema, edema, and itching in the area that had been passively sensitized. According to Gay and Chant this reaction was produced by "contralateral injection."

Ulrich (1918) and Ratner and Gruehl (1929) offer evidence which indicates that specific sensitization may develop as a result of absorption through the respiratory mucous membrane. When one considers the amount of exposure to pollen and various finely

divided material in the air, organic and inorganic compounds in food, water and the various substances with which one comes in contact, it is not surprising that allergic disease is common and that the exciting agents are numerous. In those cases in which allergy apparently occurs on the first ingestion of food, Zinsser, Enders and Fothergill (1939) suggest that active sensitization may have occurred in utero by the passage of the exciting agent from the mother through the placenta. In fact they state that there is little doubt that both active and passive sensitization of an infant can take place before birth. For some reason, however, the child is not often born hypersensitive.

Effect of Heredity on Age of Onset.—Heredity apparently influences the time of onset of allergy. Those with a bilateral inheritance are all sensitive by the age of forty, 36 per cent being sensitive before the age of five, while hypersensitive individuals with a unilateral and with negative family histories of allergy become sensitive as a rule at relatively later periods of life.

Facts About Reagin.—The antibodies or reagins which are responsible for the P-K reaction have been studied extensively. A few of the more important things that have been learned about them may be summarized as follows:

1. When a serum containing reagin is mixed with the specific atopen, the reagin titer is reduced or removed, but the capacity of the exciting agent (atopen) to elicit skin reactions remains unchanged.

2. The reaction between reagin and atopen does not lead to the formation of a visible precipitate.

3. While a very small amount of a serum containing reagin can passively sensitize the human skin, it cannot passively sensitize the skin of the lower animals.

4. Reagin is apparently more susceptible to heat than precipitins or protein sensitizers.

5. Many individuals clinically desensitized by the repeated injection of extracts of an atopen responsible for hay fever or asthma remain skin sensitive.

6. In some individuals positive skin tests can be obtained repeatedly in the same area.

7. The injection of an atopen into an atopically sensitive individual may lead to an increase in the reagin content of the blood.

8. A few cases are on record where reagin was detected in the blood of an individual before he became clinically sensitive.

9. Multiple sensitization is quite commonly observed.

10. Among individuals sensitive to food Rowe estimates that only 50 or 60 per cent will give positive skin reactions to the exciting agent.

COMPARISON OF REAGIN WITH ANTIBODIES.—It seems to us that these results are not necessarily incompatible with the view that reagin is an antibody.

According to the present concept of antigen-antibody union it is a partial filming of dispersed particles of antigen by antibody globulin; therefore, it is conceivable that the toxic property of atopen (antigen) might be retained after partial filming with reagin protein (antibody globulin). In the case of the neutralization of toxin by antitoxin the properties of the toxin are not *destroyed* since the antigenic property is unimpaired and the toxic property is demonstrable after dissociation occurs.

In regard to the reaction between reagin and atopen not leading to the formation of a visible precipitate, it is of interest to recall that Coca described an antibody which interferes with the agglutination of *E. typhosa* by its specific agglutinin. In toxin-antitoxin neutralization experiments a precipitate is formed only under certain specific conditions. In the case of syphilitic reagin a precipitate is formed when it is brought into contact with a lipid hapten only under certain conditions. Furthermore, the suppression phenomenon of Landsteiner involves the participation of an antibody in a reaction characterized by the absence of a visible precipitate. It may be that the proper conditions for a precipitin or flocculation test for atopic reagins may at some future time be discovered. In any event, it should be remembered that the phenomena of precipitation and agglutination are secondary and not primary ones in antigen-antibody reactions.

Since it has been established that immune serum from one species of animal is not always capable of passively sensitizing another species or, for that matter, every individual of the same species, it

is not surprising that human serum containing reagin will not passively sensitize the skin of every human or the tissues of the lower animals.

The apparent inactivation of reagin when heated to 56° C. for thirty minutes is not in itself a sufficient reason for omitting reagins from the list of true antibodies. Nuttall states that bacterial precipitins are inactivated at $58-60^{\circ}$ C., whereas other immune precipitins are inactivated at $68-70^{\circ}$ C. Sherman found that the apparent destruction of heterohemolysin which occurs frequently when serum containing it is heated to 56° C. for thirty minutes is only an interference or masking phenomenon. When the hemolysin is absorbed at 0° C. from the heated serum, it is found to be uninjured. At the present time Coca is inclined to agree with Zinsser that reagins may be regarded as antibodies.

Antibody Responsible for Immunity.—According to Loveless (1940) the antibody responsible for immunity to hay fever is more thermostable than reagin and unites with its homologous antigen. If the results obtained by Loveless meet with confirmation, a definite advance in our knowledge of hay fever will have been achieved.

In order to present more clearly certain additional facts about human hypersensitiveness, a few of the clinical allergies will be discussed briefly. For a more comprehensive treatment of the subject the student is referred to the monographs of Duke (1925), Alexander (1928), Coca, Walzer and Thommen (1931), Rackemann (1931), Zinsser, Enders and Fothergill (1939), Bray (1934), Vaughan (1939).

Definition of Hay Fever.—Hay fever is a term that is used quite frequently to designate all forms of vasomotor rhinitis, although Rackemann prefers to apply it only to vasomotor rhinitis caused by pollen. It will be used in this chapter in the latter sense.

Attacks of pollen hay fever are seasonal and occur only when an adequate concentration of the plant pollen to which the individual is sensitive is present in the air. The symptoms develop when the pollen comes in contact with the mucous membrane of the nose or with the conjunctiva. Since the dates of pollination of plants in most districts of the United States have been determined by botanists and specialists in allergy and are a matter of record, it is obvious that the history of the date of onset and duration of an attack of hay fever is of material value in determining the par-

ticular pollens to be used in skin tests. Since atmospheric conditions such as wind, dust and rain affect the amount of pollen in the air, it is not surprising that the patient associates such conditions with the degree of severity of his symptoms.

HISTORICAL.—The history of hay fever is quite interesting. Duke (1925) states that while cases in which headache, sneezing and itching of the nose were reported during the sixteenth and seventeenth centuries and attributed to the odor of roses, it remained for Bostock (1819) to recognize hay fever as a clinical entity. The etiology was not determined until 1856, when Blackley, an English physician, who was subject to seasonal attacks of hay fever and asthma, began his experimental study of the subject and determined that it is caused by pollen. Blackley's work was not appreciated by clinicians until it was *confirmed* by Dunbar in 1903. The results of Blackley's extensive investigation may be partially summarized as follows:

BLACKLEY'S OBSERVATIONS.—1. He tested, on himself, the pollen from approximately one hundred different species of plants and discovered that while he was sensitive to several, he reacted more severely to the pollen of rye than to other pollens.

2. In his experiments he used both dried and fresh preparations and also extracts of pollen. He employed methods involving inhalation, direct application to the mucous membrane of the nose, and instillation into the conjunctiva, and was able to produce hay fever symptoms by all of these procedures.

3. Blackley was the first one to employ skin tests. These he performed by rubbing the pollen into a scarified area of the forearm or over the tibia.

4. He made a quantitative study of the amount of pollen present on different days from early spring until August by exposing, for twenty-four hours, glass slides covered with sticky material and noting the kind and number of pollen grains that adhered to the slide. By comparing these results with his own clinical symptoms he found that the latter increased and decreased in severity as the amount of air-borne pollen to which he was sensitive was large or small. He found that the amount of pollen in the air was affected by atmospheric conditions, being considerably decreased after a heavy rain. In his case there was not sufficient pollen in the air prior to June 8 to cause an attack of hay fever. It is now generally

recognized that the June grasses are the principal causes of hay fever in the early summer, while ragweed pollen is the chief offender in August and September.

DUNBAR CONFIRMED BLACKLEY'S WORK.—The confirmation of Blackley's work by Dunbar in 1903 stimulated widespread interest in the subject. The results of subsequent research are discussed at length in the monographs of Rackemann, Coca and others. The most important discoveries are summarized earlier in this chapter.

PERENNIAL VASOMOTOR RHINITIS.—In regard to perennial vasomotor rhinitis the important facts may be presented by giving a résumé of Rackemann's report on 257 cases studied by him. The majority of these patients were young people and 73 per cent were women. In only 25 per cent did he find a history of allergy in the family. Sixteen per cent of the 257 individuals presented symptoms of asthma and 7 per cent eczema along with those of vasomotor rhinitis at the time of examination. He was able to identify the exciting agent in only 150 or 58 per cent of the 257 patients.

Perhaps the reason that such a high percentage of his cases appeared among young women is that cosmetics are used so extensively by them. He had 44 cases in which the exciting agent was orris powder. All of these gave positive skin tests. Other investigators have reported cases of vasomotor rhinitis due to rice flour and various other substances used as cosmetics. In Rackemann's series there were thirteen patients sensitive to emanations from cats, dogs, feathers, etc. In another group he places those sensitive to something in house dust.

In a few cases of vasomotor rhinitis there was an expression of hypersensitiveness to occupational dust while in others it was due to food allergy.

In 72 cases of Rackemann's series operations were performed to clear up local lesions. Tonsillectomies seemed to benefit two out of sixteen patients while four out of twenty-two were benefited by the drainage of their sinuses. The straightening of the nasal septae of thirty patients resulted in beneficial results to seven.

Asthma.—The term "asthma" according to Brown (1917) is used to apply specifically to that type of dyspnea characterized by (1) prolonged and difficult expiration especially if the attack be well developed (in the beginning of a paroxysm, however, both inspiration and expiration may appear equally difficult); (2) a

marked distention—emphysema—of the chest; (3) wheezing and sonorous sounds with both phases of respiration, but especially with expiration; (4) signs of circulatory disturbance—cyanosis and distention of the blood vessels of the neck and face; (5) secretory changes in the bronchial mucosa usually producing a considerable quantity of mucous casts, plugs, crystals, etc.; and (6) a standing or sitting posture with arms and shoulders braced so as to bring the muscles of expiration into best use."

Brown also gives an excellent discussion of the early history and theories of asthma. It has apparently been recognized clinically since the time of Hippocrates. Cullen (1788) is said to be the first to suggest "that asthma might be the result of spasm of muscles of the finer bronchi." According to Rackemann (1931) the location of bronchial obstruction in asthma may be in the larger bronchioles when the interlocking fibers of the bronchial muscles compress their openings; in the terminal bronchioles due to the contraction of true sphincters; or the bronchi may be, and perhaps always are, obstructed to a greater or less degree by the presence of a tenacious exudate within the lumen of the tube. It is definitely established that the lungs are the "shock organs" in asthma. The exciting agents are similar to those of hay fever and other allergic conditions.

Food Allergy.—Food allergy may express itself as vasomotor rhinitis, asthma, urticaria (hives), or eezema. There is a great deal of evidence that food allergy may also manifest itself as a disturbance of the intestinal tract, or perhaps by a number of other clinical symptoms. According to Rowe (1934), positive skin tests are obtained in about 50 per cent of the cases of food allergy and multiple sensitization is quite common. Piness and Miller (1931) claim a much higher percentage of positive skin tests in their series of cases. Vaughan (1930), Ellis (1931) and others have carried out extensive studies on the grouping and classification of the food allergins. They have proposed a biological classification which is regarded by many as of distinct value in interpreting skin tests and in devising "elimination diets." Vaughan states (1930) that symptoms may be caused by members of a food group to which the patient is skin negative. In his opinion sensitivity to one member of a biological food group warrants careful watching for the development of sensitiveness to

other members of the same group. Many instances of the disappearance of food allergy are on record.

The value of the botanical classification of plants yielding common food allergies suggested by Vaughan (1930) and Ellis (1931) as a basis for carrying out food testing is definitely questioned by Piness, Miller, Carnahan, Altose and Hawes (1940). In their opinion a botanical classification is an unreliable guide to predicting skin reactivity to a food when reactivity to a botanically related food is known.

Drug Allergy.—Reports of cases of hypersensitiveness to one or more of the medicinal drugs are quite numerous in the literature. As a rule the principal symptom is some form of an eruption. The latter is usually accompanied by itching. Occasionally there are dyspnea, edema, swelling of the joints and of the lymph nodes. The symptoms appear, in most cases, within a few hours after the drug has been administered. Antibodies are not demonstrable. When hypersensitiveness is once established, the patient as a rule remains sensitive for life. The development of temporary tolerance to quinine in an individual hypersensitive to quinine is reported by Hérán and St. Girons (1917). Loveman (1939) has given an excellent brief discussion of allergic drug eruptions.

Landsteiner and his associates (1935, 1936, 1938, 1940) report experimental results indicating that certain simple chemical substances may function as haptens and unite with the body protein to form new antigens, giving rise to symptoms of drug allergy or contact dermatitis, depending upon conditions.

Contact Dermatitis.—In contact dermatitis the shock organ is the epidermis. The lesions develop in the allergic individual from surface contact with the exciting agent but do not result from the injection or ingestion of the latter. The results of extensive statistical studies indicate that a Mendelian factor is not involved in this type of human idiosyncrasy. Contact dermatitis was regarded formerly as due to the action of plant poisons upon the skin and for this reason the nomenclature of dermatitis due to exciting agents in poison ivy, poison oak, etc., is misleading, since the dermatitis is a manifestation of specific hypersensitiveness to a nonantigenic excitant which is quite often soluble in fat solvents and is not due to a specific toxin.

It was established as early as 1904 by Nestler that this form of hypersensitiveness can be induced by the repeated application of the exciting agent to the skin of a nonsensitive individual. He rendered himself allergic to the juice of primrose leaves. Bloch and Steiner-Wourlich (1926) confirmed Nestler's results. Straus (1931) succeeded in sensitizing 48 babies to extracts of poison ivy leaves. In all of these experiments local sensitizing applications caused the entire skin of the individual to become sensitive. Antibodies have not been demonstrated by passive transfer. In many individuals a positive skin reaction is obtained only when the exciting agent is kept in contact with the skin for a long period of time such as occurs when the "patch test" is used. The scratch and intradermal tests are always negative except where surface contact occurs in performing the test and the individual is quite sensitive. In many cases of poison ivy, poison oak, pollen dermatitis, etc., an increased tolerance corresponding to desensitization has been produced by the injection of a 1 per cent solution of the exciting agent in sterile almond oil. Divergent results have been reported by numerous investigators. For a more extensive discussion of the subject the student is referred to the studies of Zisserman and Birch (1939), Stratton (1940), and a review of the literature by Goodman and Sulzberger (1940). References to the other papers are given in the supplementary list at the end of this chapter.

SUBSTANCES RESPONSIBLE FOR CONTACT DERMATITIS.—Coca (1931) lists a large number of substances that may cause contact dermatitis. Among them he mentioned the leaf, stalk and root of such plants as poison oak, poison ivy, poison sumac, primrose, chrysanthemum, tomato, geranium, lily and a number of others; many chemicals such as formalin, photogravure ink, hexamethylenamine, scarlach rot and various dyes, etc.; among the miscellaneous materials that have been reported as causing contact dermatitis he lists soaps, adhesive plaster, orange peel, cement, furniture polish, feathers, cereals, lanolin and a number of others. In addition to these there are on record numerous cases of contact dermatitis in individuals handling plant pollens. It is interesting to note that in pollen dermatitis the exciting agent is an oil, while in pollen hay fever it is a water-soluble substance. While these

excitants all seem to lack an antigenic property, it is quite possible that they contain specific haptens and it is conceivable that the newer work on haptens, modified antigens and specificity discussed in previous chapters may lead to a better understanding of the phenomenon of contact dermatitis.

Physical Factors in Allergy.—Duke (1925, 1932) calls attention to certain cases of clinical allergy in which the symptoms seem to indicate sensitiveness to heat, cold, or sunlight. He also reports cases which he describes as effort sensitiveness. In this connection it is of interest to note that dermatographia has long been associated with idiosyncrasy to food, although it is elicited by mechanical streaking of the skin. There is at present a difference of opinion in regard to the "physical allergies." One group of allergists maintains that the symptoms represent a sensitiveness to a physical agent, while another regards the sensitiveness to physical agents as due to physiological disturbances associated with hypersensitiveness to organic or inorganic substances. Karady (1939) suggested that a physical stimulus might alter the protein of the blood plasma in such a way as to form an "auto-antigen." Richardson (1940) has been unable to confirm Karady's work. Attention has also been called to the occasional relationship of endocrine dysfunction in skin sensitiveness to physical agents as, for example, the existence of urticaria, eczema, etc., in patients suffering from hypothyroidism (Cobb, 1919). For a recent discussion of physical allergy the student is referred to a paper by Swineford (1935).

Diagnosis of Allergy.—In the diagnosis of clinical allergy a good history is exceedingly important. When the patient is able to keep an accurate and comprehensive diary for a period that includes several attacks of allergic symptoms, the diagnosis may become quite obvious and definitely verifiable by adequate tests. Among the various other devices used by physicians in attempting to ascertain the source of the exciting agent in any particular case of clinical allergy may be mentioned the following:

a. **ELIMINATION TESTS.**—Suggestion that the patient avoid certain things that the history incriminates. This is the basis for employing elimination diets in the diagnosis of food allergies.

b. **CONJUNCTIVAL TESTS.**—In cases of pollen hay fever a dilute solution of the pollen under suspicion is dropped into the conjunctival sac. If no reaction occurs within five or ten minutes, the pollen is not incriminated. When a positive reaction occurs, it appears as a diffuse reddening of the conjunctiva accompanied by itching and a watery discharge. The intensity of the reaction can be controlled by dropping into the conjunctival sac either adrenalin (1:5,000) or cocaine (2 per cent).

c. **SCRATCH TESTS** are performed on the back, abdomen or arms of the patient. Alexander (1930) demonstrated considerable variation in skin reactions (intradermal) for different regions of the body. The skin of the back and abdomen is apparently preferable to the skin of the forearm. The technique employed by Rackemann in performing scratch tests is described by him as follows: Drops of slightly alkalized salt solution or Coea's extracting fluid are placed at least one inch apart on the skin. Powdered allergen is removed from its bottle by means of a number 4 (large size) three-cornered, straight surgical needle and stirred into a drop of solvent after thorough mixing. The needle is used to make a short scratch, about 3 mm. long, through the drop of solution. The needle is then wiped thoroughly with gauze soaked in alcohol and used to test another allergen in the next drop of solvent.

d. **PUNCTURE TEST.**—In the puncture test the reagin extract is prepared as for the scratch test. Instead of making a scratch through the drop of extract, a few skin punctures are made with the sharp point of a darning needle.

e. **INTRADERMAL TEST.**—The intradermal test is employed by many specialists in allergy. In performing this test one must employ sterile, standardized allergen extracts and preferably a separate, all-glass, accurately graduated tuberculin syringe for each extract. Rackemann employs sterile No. 26 gauge needles and injects not to exceed 0.02 c.c. of each allergen extract intradermally as in the Mantoux test.

f. **PATCH TEST.**—It has been found that in practically all cases of contact dermatitis the exciting agent will give a positive skin reaction if it is kept in contact with the skin, whereas negative results are obtained quite frequently when the scratch or intradermal

method is employed. Ramirez (1933) remarks that both Jadassohn and Block popularized the patch test in Europe while Sulzberger has stimulated interest in the test in America. In conducting the "patch test" one puts a small amount of the material to be tested on an area of healthy skin, moistens it with water if necessary, and covers it with a square of linen and then a square of rubber tissue. These are held in place by a patch of adhesive tape. When positive reactions occur they do so, as a rule, within twenty-four to forty-eight hours. Occasionally a reaction develops twenty-four hours after the material is removed. They appear as an area of redness associated with small vesicles where the material has been in contact with the skin.

g. BIOLOGICAL TEST.—Since one may be skin positive and clinically negative or skin negative and clinically sensitive to an allergen, it is often necessary to ascertain by direct experiment whether the patient is clinically sensitive to an allergen that is under suspicion. This may be accomplished by ingestion of specific foods after fasting where food allergy is under consideration; by inducing attacks through exposure to specific inhalants where the latter are under suspicion; and by natural direct contact in ascertaining the cause of contact dermatitis.

h. LEUCOPENIC INDEX.—Vaughan (1934) suggests that in food allergy it is possible to show that the ingestion of the allergen to which the patient is sensitive results in a drop in the leucocyte count (leucopenia) within fifteen minutes and a return to normal after ninety minutes. While Vaughan is apparently of the opinion that the "leucopenic index," as it is called, will prove of value in many cases of food allergy where other methods prove inadequate in identifying the exciting agent, one should remember that this test is not yet beyond the stage of experimental investigation.

EOSINOPHILES AND ALLERGY.—Auer (1915) calls attention to the development of an eosinophilia after delayed anaphylactic shock. Davison (1934) lists eosinophilia as one of the hematological findings in allergy. It is generally recognized that eosinophilic cells along with Charcot-Leyden crystals and Curschmann's spirals are quite characteristic sputum findings in bronchial asthma. Hansel (1934, 1935) has carried out an extensive study of the cytology

of the secretions of the nose and paranasal sinuses in allergy. In his opinion there is invariably a definite increase in eosinophile cells. These results are of interest when one compares allergy and anaphylaxis, since Schwenke (1912) observed an increase in eosinophile cells in the lung tissue of guinea pigs dead of anaphylactic shock. Since eosinophilia is present quite often in chorea, in syphilis and all forms of helminthiasis and in certain other pathological conditions, one wonders to what extent it is evidence of allergy. The eosinophile cell is said to be the most sensitive reagent in many bacterial infections, disappearing immediately and reappearing as the first favorable symptom. It is evident that the presence of an abnormal number of eosinophiles in abnormal secretions is presumptive but not conclusive proof that the cause of the symptoms is allergic in nature.

Mechanism of the Allergic Response.—At the present time the histamine theory discussed in the chapter on Anaphylaxis is regarded by many individuals as an adequate explanation of the mechanism involved in the allergic response. It is quite well established that the intradermal injection of a dilute solution of histamine will cause a wheal similar in appearance and cytology to that observed when a specific atopen is injected into the skin of a specifically sensitive individual. While we grant that there is a great deal of evidence that the liberation of a toxic substance occurs in the shock organ when the exciting agent reaches the latter, nevertheless, we believe that there is a serious objection to calling this substance histamine. To appreciate this objection one must recall that it is at present a well-established fact that the injection of a small amount of histamine into the body causes an increase in gastric acidity. In fact, this procedure is used quite often in the diagnosis of true achlorhydria. If histamine is liberated in clinical allergy, it would seem logical to assume that an increase in gastric acidity would occur during an attack. That such does not occur is indicated by the studies of Crip and Wechsler (1931). They determined the free HCl and total acidity of the gastric juice in forty patients and found that the concentration of each varied from zero to normal. In other words there was evidence of hypoacidity rather than hyperacidity. Others have observed an alkalosis in many patients and ketogenic diets

have been suggested. The latter are not recommended by Alexander (1928) who has made an extensive study of their value in the treatment of clinical allergy.

Abramson, Engel, Lubkin and Ochs (1938) have developed an iontophoretic method of detecting histamine in wheals of the human skin when present in dilutions of 1:5,000,000. When this method was applied to wheals produced by ragweed, timothy or ultraviolet light no histamine could be demonstrated. In a review of the literature on asthma and hay fever, Feinberg and Bernstein state that in their opinion the histamine theory of allergy cannot as yet be accepted. As a corollary to this they feel that the histaminase treatment of allergy is not yet of proved value. In this connection the observations of Campbell and Nicoll (1940) are of interest. They report the release of an active non-histamine material from sensitized guinea pig lung during *in vitro* anaphylaxis. The non-histamine substance they describe produced definite response in the rat uterus. While their results need confirmation, they should stimulate further research relative to the mechanism of the physiological response in hypersensitiveness.

Committees on Allergy Clinics.—Within recent years the growing interest in allergy has led to the organization of numerous allergy clinics. The evidence of widespread interest in allergy resulted in the appointment of committees by the Society for the Study of Asthma and Allied Conditions and the Association for the Study of Allergy, respectively, to ascertain the minimum requirements of equipment and personnel for such clinics. In their joint preliminary report made in 1932 they offer the following suggestions as to the location and equipment of an allergy clinic:

COMMITTEE RECOMMENDATIONS FOR CLINIC EQUIPMENT, SUPPLIES AND PERSONNEL.—

- “1. Room in an organized hospital and facilities for bed patients.
- “2. X-ray facilities.
- “3. Access to a nose and throat department capable of doing major sinus surgery.
- “4. Skin test materials, scratch or intradermal (should include intradermal). Facilities for keeping intradermal solutions fresh, particularly after they have been diluted.

“5. Solutions essential—minimum requirements:

Common pollen for locality	Foods:
House dust	Wheat
Orris root	Oats
Silk	Corn
Cottonseed	Buckwheat
Kapok	Rice
LePage's glue	Rye
Danders:	Egg
Horse	Milk
Dog	Beef
Cat	Lamb
Cow	Pork
Rabbit	Oyster
Sheep	Crab
Flaxseed	One common fish
Feathers:	Beans
Chicken	Potatoes
Duck	Spinach
Goose	Coconut
	Cantaloupe
	Peanut
	Celery
	Peas
	Strawberry
	Orange
	Tomato
	Chicken
	Mustard
	Chocolate

“6. Syringes and needles. At least four dozen.

“7. An adequate record system.”

In regard to personnel they recommend that anyone who does skin tests should have training and experience that is satisfactory to the Standards Committee.

Pollen Extracts.—NOON POLLEN UNIT.—According to Coca (1934) the first person to apply the principle of specific desensitization in hay fever was Noon (1911). He employed as a unit of atopen the amount present in the saline extract from one-millionth of a gram of pollen.

CONTROVERSY OVER NATURE OF EXCITING AGENT IN POLLEN.—Objections have been raised to the unit proposed by Noon, since French observed that the weight of a pollen is not always an accurate index of its content of exciting agent. There is at present definite disagreement over the nature of the exciting agent in pollen. Grove and Coca (1925) regard it as nonprotein in nature; Stull, Cooke and Tennant and their associates (1930, 1931, 1932,

1933) present evidence indicating that the exciting agents in the pollens of ragweed, timothy and certain grasses are proteins, while Black (1932) regards that of ragweed as a carbohydrate.

PREPARATION OF POLLEN EXTRACT.—To prepare a pollen extract Rackemann (1931) recommends that one gram of pollen be added to 100 c.c. of Coca's extracting fluid, the mixture shaken and allowed to stand at room temperature for three days (it should be shaken several times during this period). It is then filtered first through paper and finally through a Berkefeld N filter. According to Rackemann, nitrogen determination shows that such an extract contains approximately 0.20 mg. of nitrogen per cubic centimeter while Coca (1934) says that a 1:100 extract contains about 0.1 mg. of total N per cubic centimeter. The formula suggested by Coca for an extracting fluid for pollen and house dust is as follows:

NaCl	0.5	per cent
NaHCO ₃	0.275	per cent
Phenol	0.4	per cent

Carbon dioxide should be bubbled through this fluid until phenolphthalein added to a sample of it remains colorless. The formula for extracting fluids is varied according to the nature of the material to be extracted. These formulas are discussed in detail by Coca, Walzer and Thommen.

Rackemann suggests that during pollen hay fever season fresh extract should be made every two or three weeks and kept sealed in the ice box until ready for use. One must be certain of its sterility.

STANDARDIZING POLLEN EXTRACTS.—Various methods of standardizing extracts have been employed. As previously mentioned, Noon (1911) designated as a unit the amount of exciting agent obtained by extracting one-millionth of a gram of pollen with saline. Complement fixation has been employed, but is no longer recommended. The reaction involves titrating antigenic fractions of pollen against standardized immune serum obtained by immunizing suitable animals with pollen antigen. Since pollen possesses one or more antigenic fractions distinct from the exciting agent of pollen hay fever, it is obvious that complement fixation is a measure of antigenic fractions whose relationship to the exciting

agent is unknown. Others have suggested standardizing it by skin tests on allergic individuals, but this method has not met with favor.

Cooke and Stull (1933) standardize pollen extracts on the basis of their protein nitrogen content, while others regard total nitrogen as a better criterion. Coca (1933) offers numerous objections to the method of Cooke and Stull. He suggests (1934) a new definition of the Noon pollen unit and recommends that the Noon unit be "the quantity of pollen extract which contains 0.00001 milligram of total nitrogen." Coca says that this represents approximately the total N present in the extract of one-millionth of a gram of pollen.

Character of an Allergic Skin Reaction.—Positive skin reactions to atopens are characterized by the formation, within a few minutes after the test is performed, of a wheal one or more centimeters in diameter. The reaction consists of a reddening of the skin, which is called the flare, and the development of a central blister showing one or more pseudopods.

RECORDING SKIN REACTIONS.—In recording skin reactions some individuals describe them as + to ++++ depending upon the size of the wheal, number of pseudopods and the size of the area of erythema. According to Berkoff (1933) others record reactions as slight, moderate, marked, or very marked. Not infrequently the reactions are described by measurements. Berkoff suggests that a sheet of a modified celluloid called "absolite" or "plastocel" 9 by 16 cm., be ruled off in 1 cm. squares and that 4 eye holes 1, 1.5, 2 and 3 cm. in diameter, respectively, be made and used for measuring the size of the skin reaction. These holes represent reactions of + to ++++, respectively, and the results are so recorded.

Treatment of Allergy.—Two types of treatment apply to all forms of allergy except perhaps serum disease and these are avoidance of the exciting agent and desensitization, respectively. Experience has shown that it is not always possible to employ either of these forms of therapy and that palliative measures have to be used. The latter vary with the type of clinical allergy encountered.

The promising reports of Bloom (1938) on the use of potassium chloride and of Ertl (1939) on the use of histaminase in the treatment of allergy have not met with general confirmation. Spain,

Westcott and Gaillard (1940) report that 12 out of 15 patients with hay fever obtained no relief from symptoms when treated with potassium chloride. Feinberg and Bernstein state that a healthy skepticism should be maintained relative to the use of histaminase and of histamine in the treatment of allergy until more well-planned and carefully controlled series of cases are reported.

CORRECT BREATHING.—Brown (1925) states that in the past morphine has been the “sheet anchor” in the treatment of asthma. He recommends that asthma patients be taught to breathe slowly and quietly. In his opinion asthmatic attacks may be induced by prolonged, forced expiration and acute attacks may be relieved if the patient will let the air out of his lungs slowly and without force.

ADRENALIN AND EPHEDRINE.—It is quite customary to treat asthmatic attacks by the injection of adrenalin or by the use of some drug that will relax the bronchial musculature. Ephedrine has been used quite extensively in controlling attacks of hay fever. It should be remembered that the continued use of ephedrine and adrenalin to shrink the mucous membrane is undesirable, since the mucous membrane ultimately becomes chronically congested.

DIATHERMY.—Diathermy has been used in the treatment of hay fever and asthma with variable success. This and other methods of fever therapy are discussed by Feinberg, Osborne and Afremow (1931), Leopold and Stewart (1931) and by Miller and Piness (1931). For a more comprehensive discussion of therapy in allergy the student is referred to Rackemann (1931) or one of the other monographs mentioned at the beginning of this chapter.

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APPENDIX

COLLOIDS

Crystalloids and Colloids.—In the older literature, substances were divided into crystalloids and colloids. At the present time, it is thought that the term “colloid” should be applied to the state of the substance and that so-called crystalloids may be prepared in the colloidal state. We now speak of substances being in true solution and in the colloidal state.

True Solution.—In the case of true solutions, one is dealing with a solvent such as water and a dissolved substance, as e.g., sodium chloride, glucose, etc. When sodium chloride goes into solution in water, it tends to ionize, i.e., the molecule breaks up into sodium and chloride ions, while glucose goes into solution in the molecular state. Because of this difference, the sodium chloride tends to diffuse through a membrane more rapidly than glucose, although both are diffusible. It is estimated that the size of a molecule of sodium chloride is 5.6×10^{-8} cm. while that of glucose is 14.7×10^{-8} cm. It is obvious that the glucose molecule is considerably larger than that of sodium chloride and that it is also of a different quality.

Colloidal State.—If sodium chloride or glucose could be present in the water *not* in the *ionic* or *molecular* state but in particles or aggregates ranging in size from 0.00001 cm. to 0.0000001 cm., it would be present in the colloidal state. The water would be called the “dispersion medium” and the small particulate substance dispersed in it would be called the “dispersed phase.”

Homogeneous and Heterogeneous Systems.—In nature we find that such substances as the serum globulins and albumins and the cell protoplasm are found in the colloidal state. True solutions are also spoken of as “homogeneous systems” in which there are no apparent physical surfaces of discontinuity, while colloids are “heterogeneous systems” consisting of two or more phases separated from each other by surfaces of discontinuity. Alexander cites Zsigmondy’s observation that perhaps this basis of differentiation between homogeneous and heterogeneous systems is more

apparent than real and is probably due to the limitation of our means of observation. In any system there is a tendency for equilibrium to be established and this is of general importance in science.

The Tyndall Effect.—Another difference between a homogeneous system and a heterogeneous one containing very small dispersed particles can be demonstrated by passing a beam of light through them and making observations from a position at right angles to the beam of light. A true solution, which is an homogeneous system, should be clear, whereas the colloidal solution, which is an heterogeneous system, would appear turbid. This phenomenon is well illustrated by passing a beam of sunlight through a very small opening into a darkened room. An observer within the room can see the beam of light extending across the room owing to the presence of dust particles in the air. The light is scattered and part of it is completely polarized. The beam of light from a searchlight is also a good example of the scattering and polarizing of light by fine particles in suspension. This phenomenon, involving polarization, was first described by Faraday (1857) and later by Tyndall in 1869. It is usually called the Tyndall Effect.

Colloids are usually divided into two classes, suspensoids and emulsoids.

Suspensoids and Emulsoids.—**SUSPENSIDS.**—In these the dispersed phase is merely suspended in the dispersion medium and does not change the viscosity of the latter.

EMULSOIDS.—Here the dispersion medium and dispersed phase enter into such intimate relationship with each other that the viscosity is changed.

Transferability of Emulsoid to Suspensoid.—It is possible to convert an emulsoid into a suspensoid, as, e.g., when 50 per cent ammonium chloride is added to blood serum, which is a complex emulsoid, sufficient water is withdrawn from the serum globulin, present as a dispersed phase, to change it from an emulsoid to a suspensoid, and hence we say it is salted out.

Chemical vs. Colloid Reactions.—It is important to note that material in the colloidal state exhibits properties quite different from those when in true solution. When substances in true solutions react with each other, they do so according to known chem-

ical laws which give results that are predictable. When material is in the colloidal state, reactions do not follow the laws of mass action, etc., but are dependent largely upon the amount of surface exposed to the dispersion medium per gram of dispersed material (i.e., specific surface) and to kinetic activity of the dispersed particles. The nature of the material dispersed, as well as of the dispersion medium, is also an important factor.

Specific Surface.—The simplest example of the meaning of this term would be to take material having a density the same as water, i.e., at a standard temperature one cubic centimeter weighs one gram. In the form of a cube, the surface area of one cube would be 6 sq. cm. If this were divided into 1,000 cubes, each 1 mm. on the side, the total surface would be 60 sq. cm. If the original cube were divided into 1,000,000,000,000,000 cubes, each being 0.0001 mm. (0.1 micron) on the side, the total surface would be increased to 6,000 sq. cm. This represents what is commonly regarded as the upper limit of size of particles forming colloids. If the original cube were divided into cubes each having a length of side corresponding to the minimum of those in colloids, i.e., 1.0 millimicron or 0.001 micron, the total surface would be increased to 14.83 acres. In this case the specific surface would be per gram of dispersed material.

Surface Tension.—Wells has defined surface tension as “the force with which a fluid is striving to reduce its free surface to a minimum.” At the surface of contact of a liquid with air there is an interfacial tension and within a colloid there exist interfacial tensions where the dispersion medium is in contact with the surface of the dispersed material. This can, perhaps, be understood by visualizing not a colloid but a drop of fat added to hot water. It appears as a flattened sphere floating on the surface because the attraction of the fat molecules for each other is greater than the attraction of the surface water molecules for the fat. In this case, part of the fat is exposed to air and part to water. The force or tangential pull that maintains the surface of the oil droplet exposed to air is its surface tension in respect to air, while the force that maintains the integrity of the surface in contact with water is its surface tension with respect to water. Thus the student should be able to appreciate that at the surfaces of contact between two substances there exist interfacial tensions. Ordinarily the sur-

face tension of a medium is measured against air. In the colloidal state each colloidal particle is exposed at its surface to the dispersion medium and there exists at the surface a tangential force which maintains the integrity of the surface and attempts to reduce the surface area to a minimum and this is the interfacial tension.

Electrical Phenomena and Surface Potentials.—The terms “membrane potential” and “critical potential” have, during recent years, crept into immunological papers having to do with agglutination, precipitation and complement fixation. It is hoped that the following simple explanations may help the student in his reading of current literature.

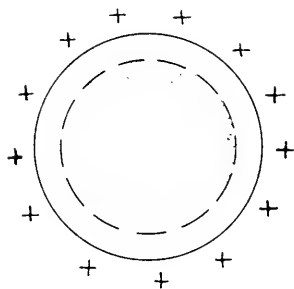


Fig. 24.

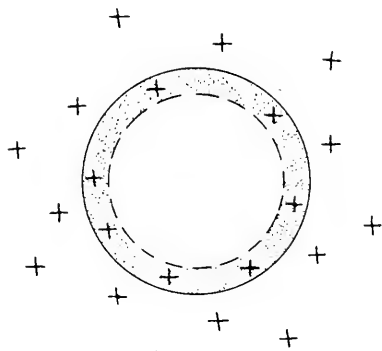


Fig. 25.

Fig. 24.—Negatively charged particle surrounded by single layer of positive charges, theory of Helmholtz.

Fig. 25.—Negatively charged particle surrounded by diffuse atmosphere of positively charged particles, theory suggested by Gouy.

ELECTRICAL DOUBLE LAYER, HELMHOLTZ.—In colloidal solutions the dispersed particles are conceived of as being somewhat like a condenser in that at the surface there is a double layer of opposite electrical charges. One layer is within the wall of the particle and the layer of opposite charges is at a molecular distance away but in the dispersion medium. This double layer conception was originated by Helmholtz. More recently the outer layer is conceived of as forming a diffuse atmosphere rather than a single layer. These two concepts are illustrated in Figs. 24 and 25 which show the particle to be negatively charged and surrounded with a simple or diffuse atmosphere of positive charges.

MEMBRANE POTENTIAL.—It will be observed that the outside layer has the same total electric charge as the inside layer although opposite in kind. There is then a difference in potential between those two layers. The stability of colloids runs parallel with the drop in the potential in the double layer. This drop in potential is due to changes in the outside layer. It is now known that this layer is more complex than has been indicated here and that one portion only of the total potential difference is important in colloid stability.

Effect of Cations on Colloids.—It has long been known that free hydrogen ions in the dispersion medium profoundly affect the stability of colloids. When their concentration is sufficient to bring about complete neutralization of charge, the colloid becomes very unstable and is readily precipitated. This is called the isoelectric point. This is in accordance with the observation that colloidal particles having like charges repel each other.

Critical Potential.—The point in the drop in potential where the colloidal particles barely repel each other is called the critical potential, a fall below which indicates the loss of the property of repulsion. This discussion applies more nearly to suspensoids than to emulsoids. In the latter group of colloids the dispersion medium enters into intimate relationship with the dispersed material.

Factors Governing Stability of Emulsoids.—It is thought that there are at least two factors involved in maintaining the dispersed state of emulsoids. One factor is the charge and potential and the other is the so-called film of water around each particle. Removal or reduction in amount of this water film favors precipitation. If an adequate water film is maintained, a drop in potential may not lead to precipitation. This will be more fully discussed under Cohesion and Precipitation.

Migration of Charged Particles.—**CATAPHORESIS.**—As is evident from the preceding discussion, one of the important properties of colloids is that the dispersed particles are electrically charged with respect to their surroundings. When an electric current is passed through a colloid, any negatively charged particles will travel toward the positive pole, or anode, while positively charged particles will travel toward the cathode or negative pole. This migration phenomenon is called cataphoresis. If the dispersed particles have

lost their charge, they will not travel to either pole. This state of affairs exists at the "isoelectric" point where neutralization of charge has occurred. By cataphoretic experiments the nature of the charge of various colloids has been determined.

Adsorption.—**ADSORPTION AND SURFACE AFFINITIES.**—It is generally known that finely powdered charcoal will remove colored substances that are present in a solution. The intensity of color of certain dye solutions is markedly reduced by charcoal. In each case, the material removed has attached itself to the surface of the finely dispersed particles of charcoal. This phenomenon is called adsorption and is a characteristic of all matter in the colloidal state.

Positive Adsorption.—The term positive adsorption means that there has occurred an increase in concentration of some substance in the boundary layer of the dispersed substance and dispersion medium.

Surface Concentration of Dissolved Substance.—Willard Gibbs called attention to a principle that is perhaps fundamental to adsorption, and that is, that when a substance is dissolved in a liquid and lowers its surface tension, it appears in greater concentration at the surface. It is now generally thought that the way the surface molecules are oriented is responsible for many of the properties of adsorption.

Surface Wetting.—It is readily appreciated that when oil or fat is dispersed in water the water is unable to wet the greasy surface of the dispersed fat droplets. On the other hand, if oil droplets can be filmed with proteins, the surfaces may readily be made wet with water.

DEVAUX EXPERIMENT.—Alexander cites an experiment of Devaux which seems to explain the phenomenon of surface wetting. Devaux floated a drop of molten fatty acid on the surface of some hot water and allowed it to cool. The upper surface was in contact with the air, while the lower was adjacent to the water. He found that after he dried the lower surface he could wet it with water but that he could not wet the upper surface with water. Alexander says that apparently the "hydrophile" or "friendly-to-water" ends of the fatty acid molecules were turned toward the surface that cooled in contact with the water, while the "hydrophobe" or

“greasy” ends were turned outward in the surface which had cooled in contact with the air.

MOLECULAR ORIENTATION. HARKINS’ THEORY.—This surface orientation of molecules is part of Harkins’ orientation theory. He would say that on the surface wet by water, the molecules were oriented at right angles to the surface with a polar group attached to a short carbon chain and that this polar group attracted the water so strongly that it tended to drag the hydrocarbon chain out into the water. This affinity for water was responsible for the wetting. On the side exposed to air, the other end of the chain was outside and it had no affinity for water, hence, the surface

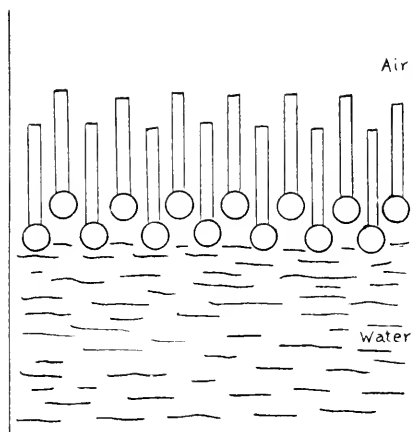


Fig. 26.—Orientation of molecules of an alcohol (or an organic acid) at the surface of its aqueous solution. From “Atoms, Ions, Salts and Surfaces,” by William D. Harkins in *Newer Knowledge of Bacteriology and Immunology* edited by E. O. Jordan and I. S. Falk. Reprinted by permission of the University of Chicago Press.

could not be wetted. Langmuir has studied this phenomenon of molecular orientation and arrived at the same conclusions as Harkins. This surface orientation of molecules is graphically illustrated by Harkins. (Fig. 26.)

Attraction of Colloids for Each Other.—**COHESION, ADHESION AND PRECIPITATION.**—Graham noted that colloids adhere to each other with great tenacity. Within a colloid there are powerful residual forces that hold adjacent molecules together. At the surface there are unsatisfied fields of force that make it possible for the dispersed particles to stick together if the forces holding them

apart are not sufficient. Every colloid is a heterogeneous system in which there is a continual effort toward establishing equilibrium.

Fundamental Problem of Colloid Chemistry.—Kruyt says that the fundamental problem of colloid chemistry is to ascertain the reason for the inability of the surface tension to unite the particles. In the case of suspensoids it seems that the surface charge and difference in potential are fundamental factors in keeping the particles apart, while the orientation of surface molecules is fundamental in determining the cohesive or adhesive forces.

In emulsoids, conditions are a little more complex and a third factor perhaps ranks first in importance. Attention has previously been called to the difference between emulsoids and suspensoids. In emulsoids, the dispersion medium forms an intimate relationship with the dispersed material. Since the emulsoids encountered in immunity are made up of water as the dispersion medium and protein the dispersed substance or phase, the water is in intimate relationship with the dispersed particles of protein. It is considered that each particle has an intimate water envelope around it.

Factors in Emulsoid Stability.—The factors involved are the particle and its surface, the water layer and the electrical double layer. Anything that reduces this water layer to a certain critical amount increases the cohesive properties. The lowering of the potential to the critical point favors precipitation and also cohesion, and as mentioned above the nature of the material of the surfaces is an additional factor. The water envelope is of sufficient importance to prevent precipitation even when the critical potential is reached. This is illustrated by Kruyt (Fig. 27).

Precipitation by Electrolytes.—It will be seen from Fig. 27 that if the charged emulsoid is dehydrated, it becomes a charged suspensoid that can be precipitated by electrolytes neutralizing the charges. If the charge on the emulsoid is removed by electrolytes, it remains an emulsoid, but without a charge, and can be precipitated by dehydrating it with alcohol. Thus it will be seen that the neutralization of charge causes precipitation or coagulation of suspensoids but not of emulsoids.

Macleod says that "a quantity of electrolyte which is capable of producing complete precipitation when added all at once to suspensoids will be ineffective when added in small quantities. This

phenomenon, which is exhibited when toxins and antitoxins are mixed together, is probably due to the fact that precipitation depends on inequality and irregular distribution of electric charges, a condition which becomes established when the electrolyte is suddenly added, but not so when it is gradually added." This phenomenon is of considerable importance in immunology.

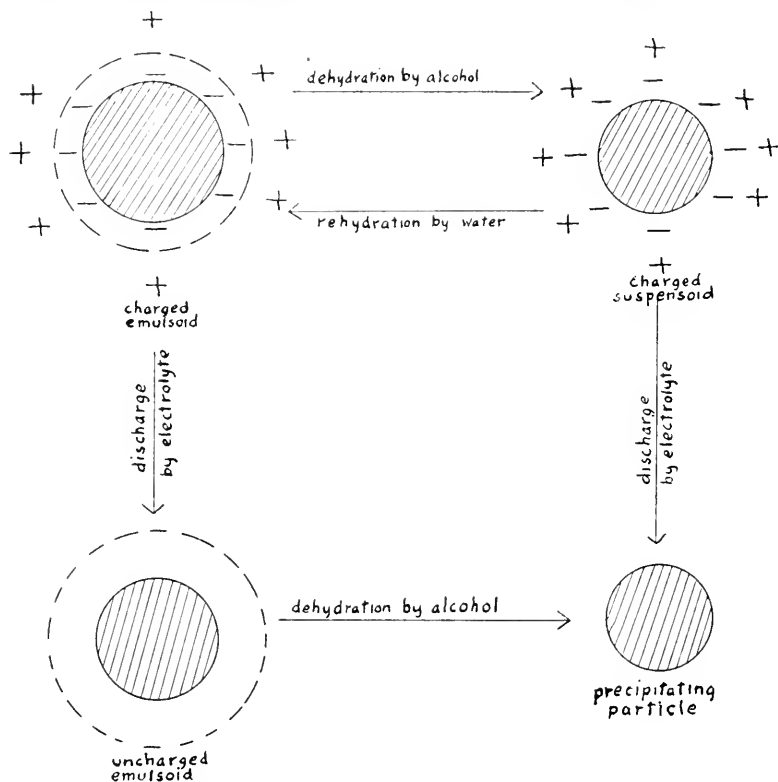


Fig. 27.—Factors involved in dispersion and precipitation of colloids. (After Kruyt and Van Klooster, *Colloids*, John Wiley and Sons, 1930.)

Protective Action of Colloids.—From the preceding discussion it can be seen that emulsoids are much more resistant to the precipitating action of electrolytes than suspensoids. This is due to their water layer. If the dispersed particles of a *suspensoid* like colloidal gold, which is quite sensitive to the precipitating power of electrolytes, can be made to adsorb a film of a stable emulsoid around each particle, then the suspensoid will be pro-

ected from the precipitating action of electrolytes. It has acquired the stability of the adsorbed emulsoid.

Autoprotection.—Autoprotection, double and plural protections have been observed. As an example of autoprotection, Alexander mentions that in pure iron “one allotrope, γ - iron, seems to be adsorbed by α - iron.”

Effect of Speed of Mixing.—When oppositely charged colloids are mixed, it is quite possible for flocculation to occur as a result of the neutralization of charges. If, however, an excess of a positively charged emulsoid is added to a negatively charged suspensoid, the emulsoid may be adsorbed before precipitation occurs. If a small amount of the emulsoid instead of an excess is added, or if the emulsoid is added very slowly, precipitation may occur before the protection is established. This shows that relative proportions and speed of mixing profoundly affect colloidal reactions. This is commonly given as the explanation of the zoning phenomena seen in many immunological reactions. It can also be shown experimentally that when small amounts of the protective colloid added bring the other colloid to the isoelectric point, precipitation commonly occurs.

Solubility of Precipitates.—In regard to protection and protectors, Alexander* says, “although some salts (citrates, sulphyocyanates) may act as protectors, protection is generally accomplished by adding a reversible or emulsoid colloid to an irreversible one, which thereupon acquires reversible properties, that is, it becomes insensitive to electrolytes, redissolves after desiccation (at any temperature that does not render the protector insoluble) and passes through ultrafilters that would otherwise hold it back.”

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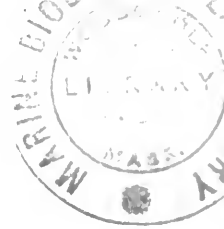
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